Molecules and Cells

Minireview

Regulator of Calcineurin (RCAN): Beyond Down Syndrome Critical Region

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The regulator of calcineurin (RCAN) was first reported as a novel gene called DSCR1, encoded in a region termed the Down syndrome critical region (DSCR) of human chromosome 21. Genome sequence comparisons across species using bioinformatics revealed three members of the RCAN gene family, *RCAN1*, *RCAN2*, and *RCAN3*, present in most jawed vertebrates, with one member observed in most invertebrates and fungi. RCAN is most highly expressed in brain and striated muscles, but expression has been reported in many other tissues, as well, including the heart and kidneys. Expression levels of RCAN homologs are responsive to external stressors such as reactive oxygen species, Ca^{2+}, amyloid β, and hormonal changes and upregulated in pathological conditions, including Alzheimer's disease, cardiac hypertrophy, diabetes, and degenerative neuropathy. RCAN binding to calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase, inhibits calcineurin activity, thereby regulating different physiological events via dephosphorylation of important substrates. Novel functions of RCANs have recently emerged, indicating involvement in mitochondria homeostasis, RNA binding, circadian rhythms, obesity, and thermogenesis, some of which are calcineurin-independent. These developments suggest that besides significant contributions to DS pathologies and calcineurin regulation, RCAN is an important participant across physiological systems, suggesting it as a favorable therapeutic target.

Keywords: calcineurin, Down syndrome, RCAN1, RCAN2, RCAN3

INTRODUCTION

The regulator of calcineurin (RCAN) was first reported as a Down syndrome critical region 1 (DSCR1), which is encoded in a region that at that time was thought to participate in the onset of Down syndrome (DS) (Antonarakis, 2017; Fuentes et al., 1995). Soon after, evidence showed that RCAN binds to and regulates the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin, whose substrates include nuclear factor of activated T cells (NFAT), the transcription factor that regulates gene expression in many cell types, including immune cells (Kingsbury and Cunningham, 2000; Wu et al., 2007). In this context, RCANs are reported to either facilitate or inhibit calcineurin, depending on RCAN protein amount and calcineurin affinity (Li et al., 2016; Martínez-Høyer et al., 2013; Mehta et al., 2009; Shin et al., 2006; Vega et al., 2003). Although most invertebrates, protozoa, and fungi encode a single RCAN, three *RCAN* genes, *RCAN1*, *RCAN2*, and *RCAN3*, are reported in Gnathostomata, each of which codes multiple transcriptional and translational products (Serrano-Candelas et al., 2014) (Table 1, Fig. 1). Because of RCAN's broad and inducible expression under stress-related circumstances across tissues, and complex gene expression patterns due to multiple RCAN genes, RCAN was originally associated with a suite of different names according to function and

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Table 1. Human RCANs (Serrano-Candelas et al., 2014)

	RCAN1			RCAN2			RCAN3		
Gene location	Chromosome 21 ACD 21 cluster 21q22.12			Chromosome 6 ACD 6 cluster 6p12-21			Chromosome 1 ACD 1 cluster 1p35		
Other names	DSCR1 MCIP			ZAKI-4			DSCR1L2 (protein)		
Transcripts[a]	*RCAN1-1*		*RCAN1-4*	*RCAN2-1*	*RCAN2-2*	*RCAN2-4*	*RCAN3-1*	*RCAN3-2*	*RCAN3-3*
Translation products	RCAN1-1L[b] 252 aa	RCAN1-1S[b] 197 aa	RCAN1-4 197 aa	RCAN2L/ RCAN2-3/ RCAN2β[c] 243 aa		RCAN2S/ RCAN2-4/ RCAN2α 197 aa	RCAN3/RCAN3-4[c] 241 aa		
References	(Fuentes et al., 1995; Rothermel et al., 2001)			(Cao et al., 2002; Miyazaki et al., 1996)			(Facchin et al., 2011; Strippoli et al., 2000)		

[a]Only most-studied products are shown.
[b]Alternative translation products.
[c]Only one protein product is produced from the multiple transcripts.

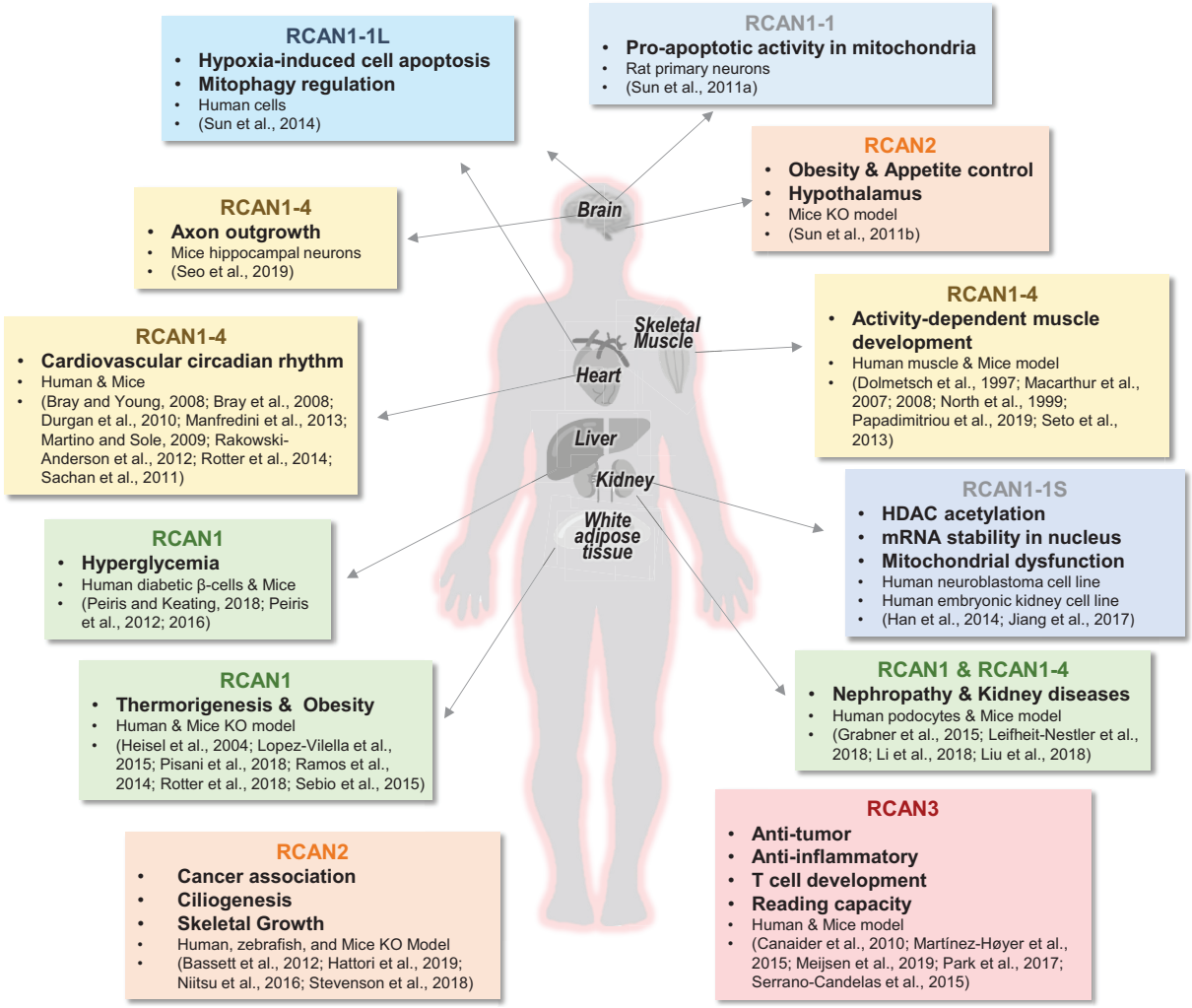

Fig. 1. RCAN functions (see Table 1 and Fig. 3). RCANs are expressed in various tissues and organs and participate in specialized roles in gene expression, development, metabolism, and behaviors. RCANs are detected not only in cytoplasm but also in subcellular organelles including mitochondria and nucleus.

context, including ZAKI, MCIP, and Adapt78 (Crawford et al., 1997; Kingsbury and Cunningham, 2000; Miyazaki et al., 1996; Yang et al., 2000). Moreover, different nomenclature for RCANs, including *Sarah*, *Nebula*, *Rcn*, CBP, and calcipressin, apply to different invertebrate model systems (Chang et al., 2003; Ejima et al., 2004; Görlach et al., 2000; Lee et al., 2003). RCAN was thereby proposed as a root name to preclude confusion and facilitate research across organisms and fields (Davies et al., 2007). Despite this effort, DSCR1 is still frequently used to emphasize RCAN's importance in DS.

Among the three human RCAN genes, RCAN1 is located on chromosome 21, trisomy of which (T21) causes DS (Fuentes et al., 1995). Although neither ubiquitous nor constitutive, RCAN1 is nonetheless highly expressed in many different tissues, including the brain and heart (Fuentes et al., 2000). RCAN1 overexpression due to triple copies of the gene contributes to mental retardation and congenital cardiac defects, the hallmarks of DS, hindering dephosphorylation of many different important physiological substrates of calcineurin such as ion channels and transporters, mitochondrial function regulators, and NFATs (Harris et al., 2005; Roy and Cyert, 2019). Besides DS, the expression of RCAN1 is increased in other clinical conditions, including Alzheimer's disease, cardiac hypertrophy, and diabetes, all of which are often associated with DS (Jeong, 2017; Peiris and Keating, 2018). RCAN1 is upregulated by stress factors, such as protein aggregates, elevated intracellular calcium, oxidative stress, and glucocorticoid, prevents excess and dangerous over-activation of calcineurin.

RCAN is conserved across fungi and animals, facilitating isolation of RCAN as an endogenous regulatory partner of calcineurin through molecular genetic screening in budding yeast or pathogenic fungi (Görlach et al., 2000; Kingsbury and Cunningham, 2000). Genetic studies using many different model systems with vertebrates and invertebrates alike have shown that although RCAN localizes to some subcellular organelles, such as nuclei and mitochondria, it is predominantly found in the cytoplasm (Chang and Min, 2005; Sun et al., 2011a). A bioinformatics mining of an extensive molecular dataset suggested RCAN participates in a range of molecular and cellular processes as a regulator of many physiological activities, leading to intriguing questions as to RCAN's multi-functionality. This review discusses RCAN discovery and its activity in DS, and recent developments in delineating RCAN1 function across multiple physiological and pathological contexts (Figs. 1 and 2).

RCAN DISCOVERY

RCAN1 was first reported as the coding gene DSCR1 located on human chromosome 21 in a region thought to be a minimal region responsible for pathological phenotypes of DS (Fuentes et al., 1995). RCAN1 was predicted to be involved in transcriptional regulation and signal transduction based on an amino acid sequence that contains many acidic and proline-rich domains. In a separate study, ZAKI-4 was designated as a thyroid hormone-responsive gene expressed in human skin fibroblasts completely separated from DSCR1, which had yet to be added to any shared database (Miyazaki et al., 1996). Follow-up studies revealed that ZAKI-4 is one of a family of RCAN proteins, and it was later confirmed as RCAN2, the homolog encoded in chromosome 6 (Cao et al., 2002; Siddiq et al., 2001). Subsequent studies revealed that Adapt78, a factor strongly upregulated by hydrogen peroxide treatment in hamster ovarian fibroblasts, was also RCAN1, suggesting that RCAN1 protects cells from oxidative damage (Crawford et al., 1997).

Concurrent studies were conducted to identify inhibitory regulators of calcineurin, taking advantage of fungi as a model because of their versatile molecular genetics. Kingsbury and Cunningham screened a genomic library for genes that confer Ca^{2+} tolerance and uncovered a null mutant of Pmc1, a P-type Ca^{2+}-ATPase, in *Saccharomyces cerevisiae* (Kingsbury and Cunningham, 2000). The restoration of Ca^{2+} tolerance in *pmc1*Δ with a plasmid containing the open reading frame for Rcn1, a yeast RCAN1, was found to link to Vcx1p, a vacuolar H^+/Ca^{2+} exchanger. Vcx1p is inactivated by calcineurin blockers, such as FK506 and cyclosporine A (Cunningham and Fink, 1994; 1996). Two-hybrid screening in *Cryptococcus neoformans*, an opportunistic pathogenic fungus, also indicated that RCAN1 was CBP1/calcipressin and strongly bound to calcineurin A and B subunits to inhibit phosphatase activity (Görlach et al., 2000). Rothermel et al. (2000) reported that RCAN1 suppressed calcineurin-dependent transcription in a cell line of mouse skeletal myoblasts based on previous observations of DSCR1, ZAKI-4, and an ongoing study of the yeast RCAN1 and proposed use of the term MCIP, myocyte-enriched calcineurin interacting protein, to define the protein.

High conservation of RCAN1 across species supports a genetic approach to delineation thereof using established experimental models. Transgenic mice overexpressing RCAN1 under control of a cardiac-specific promoter present with smaller hearts and suppressed cardiac hypertrophy caused by expression of constitutively active calcineurin (Rothermel et al., 2001). The RCAN1 protein in *Caenorhabditis elegans* shares 40% amino acid sequence identity with the human homolog identified as RCN-1, which is later renamed as RCAN-1 according to the new nomenclature (Davies et al., 2007). RCAN-1 is highly expressed in many different tissues, including muscle and neurons, and inhibits mammalian calcineurin phosphatase activity (Lee et al., 2003; Li et al., 2016). Overexpression of RCAN1 in worms recapitulates phenotypes observed in calcineurin-deficient mutants, such as small body size and defective behavior; these phenotypes are rescued in calcineurin gain-of-function mutants expressing a constitutively active form of RCAN-1 (Lee et al., 2003). An RCAN1 homolog in *Drosophila melanogaster* was termed Nebula, and *nebula* loss-of-function and overexpression mutants exhibit learning defects (Chang et al., 2003). Fly RCAN1 was also isolated and termed *Sarah (sra)* in a subsequent genetic screen for genes responsible for female fertility (Ejima et al., 2004; Horner et al., 2006). These studies utilizing genetic models help understand tissue-specific functions of RCAN1 at organismal level in different contexts of development and behavior.

RCANs were logged under more than 20 different names from the late 1990s to the early 2000s. Most are rooted in one of the following: DSCR, Rcn, ZAKI, CBP1, calcipressin, or

MCIP, and most are numbered according to different transcripts and translational products resulting from differential transcription, splicing, and translation events. The term RCAN was then introduced to encompass RCAN family members and provide a coherent naming system (Davies et al., 2007) (Fig. 2, Table 2). Although this nomenclature was widely

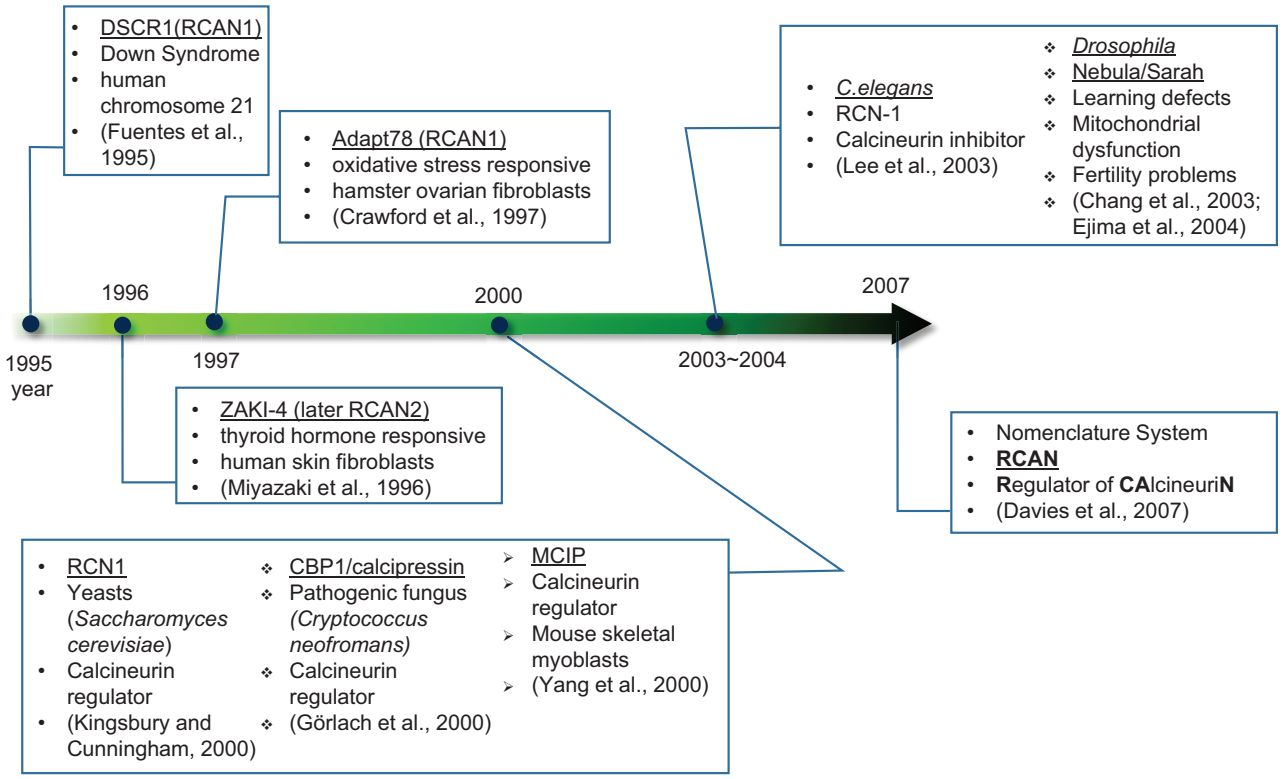

Fig. 2. Discovery and nomenclature of RCANs. Since identified as a novel gene in human chromosome 21 and named DSCR1 in 1995, RCANs are reported multiple times in different names and different species, including yeasts, pathogenic fungus, worms, flies, mice, and human cells. In 2007, finally, a new nomenclature system was proposed to use RCAN.

Table 2. RCANs in non-vertebrates

Species	Yeast (*S. cerevisiae*)	Pathogenic fungus (*C. neoformans*)	Fly (*D. melanogaster*)	Nematode (*C. elegans*)
Common names	RCN1	CBP1 (calcineurin binding protein 1) Calcipressin	Nebula, Sarah	RCAN-1 RCN-1
Phenotypes				
Null mutants	Low calcineurin activity	Slow growth Low virulence Mating failure	Mitochondrial dysfunction Learning defect Fertility defect Courtship problem	Cryothermotaxis Sensory dysfunction
Transgenics	Calcineurin inhibition	N/A	Mitochondrial dysfunction Learning defect	Slow growth Small body Abnormal behavior
References	(Kingsbury and Cunningham, 2000)	(Görlach et al., 2000)	(Chang and Min, 2005; Chang et al., 2003; Ejima et al., 2004; Horner et al., 2006; Shaw and Chang, 2013; Shaw et al., 2015; Takeo et al., 2006)	(Lee et al., 2003; Li et al., 2015; 2016)

N/A, not available.

adopted, some reports still use older terms, of which DSCR is the most frequent, possibly because of its importance in DS. Recent reports of differential RCAN functions indicate that RCAN is critically involved in multiple cellular processes besides its DS involvement. Recent evidence also indicates a lack of a single discrete region of "DSCR" in human chromosome 21 responsible for the onset of DS (Antonarakis, 2017; Letourneau et al., 2014; Lyle et al., 2009). RCAN thereby reflects current knowledge as to its predominant molecular function.

LACK OF A SINGLE DS REGION OR GENE

Evidence from the late 1950s and early 1960s showed that DS also occurs in cases of partial T21 following translocation of portions of chromosome 21. Investigations thus followed on specific regions of chromosome 21 to uncover genes whose overexpression leads to DS symptoms (Deutsch et al., 2005; Mégarbané et al., 2009). Molecular genetic analyses of partial T21 in the late 1980s and the early 1990s identified 21q21 to 21q23 as a plausible minimal region that, when triplicated, caused DS phenotypes and was termed the Down syndrome critical region (DSCR) at that time (Delabar et al., 1993; Korenberg et al., 1990; McCormick et al., 1989; Rahmani et al., 1990). These early analyses were impeded by low-resolution mapping techniques on a limited number of cases because of the rare incidence of partial T21 and different combinations of pathogenic phenotypes. Moreover, the triplicated region detected in each affected individual varied in size and location, confounding investigations of the contribution of other regions, and delineation of a particular region or regions responsible for a given DS phenotype. Development of advanced mapping techniques and increasing numbers of DS cases revealed that microduplications of only a part of the DSCR, triplication outside the DSCR, and even partial monosomy of chromosome 21 inside or outside the DSCR all resulted in DS symptoms (Antonarakis et al., 2004; Lyle et al., 2009). Researchers have since concluded that there is no single discrete region of chromosome 21 implicated in DS.

Current, widely accepted explanations for the pathology of DS now focus on genomic dosage imbalance, following modifications collected through decades of accumulating evidence (Antonarakis et al., 2004). The latest studies indicate four types of genetic elements present in chromosome 21 associated with DS onset or a lack thereof: a dosage-sensitive protein-coding gene that contributes to the T21 phenotype, a dosage-insensitive protein-coding gene that does not, a non-protein-coding functional regulatory region, and a neutral noncoding region (Antonarakis, 2017). Dosage-sensitive genes could affect DS pathology via direct and indirect effects on encoded protein products, such as allele specificity, or different combinations of alleles (Mégarbané et al., 2009).

The search for a gene or group of genes with dose expression levels in direct association with specific DS phenotypes has revealed that only some genes on chromosome 21 are overexpressed at the theoretical value of 1.5 fold (for euploidy) in DS, and of these, many are compensated for elsewhere (FitzPatrick et al., 2002). Studies have also shown that the expression levels of genes on other chromosomes are dysregulated in DS, as well, potentially as a consequence of inter-chromosomal gene interactions involving T21 (Dahoun et al., 2008; Letourneau et al., 2014). Those gene expression dysregulation domains (GEDDs) on chromosomes in T21 include genes that are closely associated with mental retardation such as fragile X syndrome (Han et al., 2015; Zhang et al., 2019). Change in gene expression pattern such as GEDDs in DS can be also tissue-specific (Lee et al., 2019). These studies all indicate that DS phenotypes result from the combinatorial effect of gene expression pattern associated with chromosome 21 anomaly.

Comparative studies using systematic gene expression profiles are improving our understanding as to which genes are more strongly associated with certain phenotypes than others (Grossman et al., 2011; Lane et al., 2014). Some evidence suggested that genes exhibiting haploinsufficiency may also be sensitive to overexpression of T21 (Antonarakis, 2017). A transcriptomic comparison between monozygotic twin fetuses, only one of whom was T21, showed that differentially regulated gene expression between the twins was organized in domains along all chromosomes (Letourneau et al., 2014). Gene expression dysregulation patterns pointed to modifications of the chromatin environment in the nuclear compartments of trisomic cells that influenced the entire transcriptome. Researchers have come to a consensus that there is no single gene responsible for DS, which rather results from multiple effects, including those associated with an additional copy of chromosome 21 and those resulting from altered gene expression of other chromosomes via inter-chromosomal interaction and epigenetic modification.

RCAN IN DOWN SYNDROME

The RCAN1 gene on chromosome 21 is reported to be overexpressed across tissues in DS, including the brain, heart, and skeletal muscle (Fuentes et al., 2000). These effects have been associated with the three copies in T21 and a single copy in partial monosomy 21, presumably via interactions between chromosomes and epigenetics (Antonarakis et al., 2004; Letourneau et al., 2014; Lyle et al., 2009). A genotype–phenotype comparison study on a case of a very small partial monosomy 21, comprising 1.48 Mb and containing only eight genes, including RCAN1, showed a severe phenotype with mental retardation, short stature, and cardiac anomaly, implicating RCAN1 in pathological states of DS (Lyle et al., 2009). This finding is confounded, however, by the discovery of an approximately 500 kb region of human chromosome 21 containing RCAN1 replicated on chromosomes 6 and 1 to generate RCAN2 and RCAN3, suggesting two evolutionary, whole-genome duplication events and subsequent selective deletion events further along the evolution timeline (Ohno, 1970; Serrano-Candelas et al., 2014; Strippoli et al., 2002). RCAN1 is located at 21q22.12, RCAN2 at 6p12-p21, and RCAN3 at 1q35-q36. All of the three genes contain seven exons and encode multiple transcripts and translated products via alternative promoters, splicing, and usage of different initiation codons (Serrano-Candelas et al., 2014; Wu and Song, 2013). All known RCAN proteins contain C-terminal calci-

neurin interacting motifs, suggesting that all RCAN proteins can influence calcineurin activity, although whether they all do so or not remains inconclusive. Extricating how different members of the RCAN family specifically contribute to DS is even more confounded by the fact that DS gene dosage imbalance has been found across the entire genome. However, *in vivo* studies have shown that adjusting RCAN1 expression levels to within normal ranges in DS cells can rescue certain pathological phenotypes, even though expression patterns of different RCAN proteins may vary across DS presentations. In human DS organs and murine models, sympathetic neurons lose their innervations during development because of impaired neurotrophin trafficking. Patel et al. (2015) found that in a mouse model of DS, correcting the copy number of the RCAN1 gene rescued this defective phenotype. Delayed differentiation of neocortical progenitor cells in the brains of DS mouse embryos was also ameliorated by knockdown of RCAN1 (Kurabayashi and Sanada, 2013). Transgenic mice overexpressing RCAN1 develop abnormal hippocampi that are unable to maintain long-term potentiation, which has been associated with deficits in learning and memory (Martin et al., 2012). Altogether, research on RCAN family protein functions in different physiological contexts suggests complicated involvement in multiple pathologies.

RCAN FAMILY PROTEINS AND FUNCTIONS

The genes encoding the three RCAN isoforms generate five major protein-producing transcripts, *RCAN1-1*, *RCAN1-4*, *RCAN2-1*, *RCAN2-3*, and *RCAN3*, with the corresponding protein products RCAN1-1L, RCAN1-1S, RCAN1-4, RCAN2L, RCAN2S, and RCAN3 (Table 1, Fig. 3). RCAN isoforms are differentially expressed in several tissues, including the brain, heart, skeletal muscle, kidney, and intestine, with functions associated with many pathologic conditions via multiple molecular and cellular mechanisms.

RCAN1

RCAN1 isoforms: Abundant RCAN1-1 and Inducible RCAN1-4

Transcriptome data from the National Library of Medicine database indicate that RCAN1 transcripts are abundant in many different tissues such as the brain, heart, kidney, liver, thyroid,

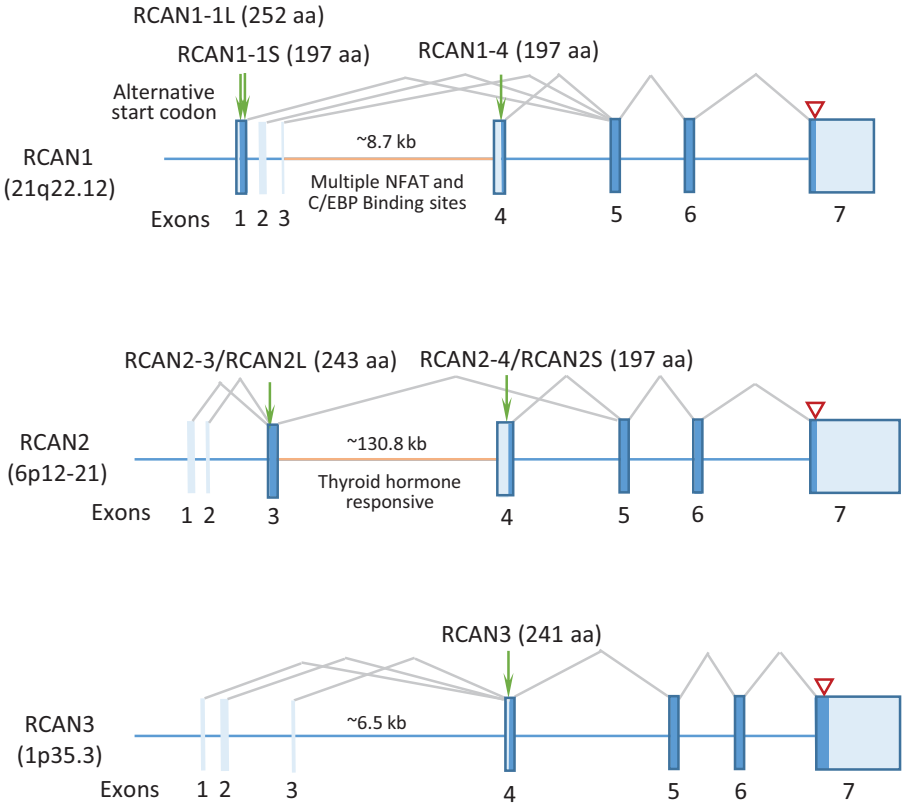

Fig. 3. Human RCAN Family members. RCAN genes contain seven exons. RCAN1-1 transcripts (exons 1,5,6,7) produce RCAN1-1L (252 aa) and RCAN1-1S (197 aa) using different start codons in exon 1, while RCAN1-4 transcripts (exons 4,5,6,7) do RCAN1-4 protein (197 aa). RCAN2-1 or RCAN2-2 transcripts (exons 1,3,5,6,7 or exons 2,3,5,6,7) produce RCAN2-3/RCAN2L (243 aa) and RCAN2-4 Transcripts (exons 4,5,6,7) produce RCAN2-4/RCAN2S (197 aa). All RCAN3 transcripts, RCAN3-1, -2, and -3 (exons 1,4,5,6,7; 2,4,6,7; 3,4,5,6,7), produce only one translational product of RCAN3 (241 aa). Green arrows indicate start codons and open red arrowheads point stop codons. Introns containing promoters for the transcripts starting exon 4 are shown in orange. Untranslated regions are in light blue. NCBI database (https://www.ncbi.nlm.nih.gov/gene/) indicates that other transcript variants are detected in various RNA-seq datasets, but their predicted translational products have not confirmed.

pancreas, urinary bladder, gall bladder, and placenta (National Center for Biotechnology Information of National Library of Medicine; https://www.ncbi.nlm.nih.gov/). The most studied RCAN1 isoforms are RCAN1-1 and RCAN1-4, produced by alternative splicing of exons 1 and 4, respectively, to exons 5 to 7 (Ermak et al., 2006; Fuentes et al., 1997). Two isoforms, RCAN1-1L (long) comprising 252 amino acids, and RCAN1-1S (short) comprising 197 amino acids, are alternatively translated using different start codons on the RCAN1-1 transcript (Genescà et al., 2003; Wu and Song, 2013). The RCAN1-4 protein contains 197 amino acids. All of these isoforms share the 168 amino acids at the C-terminal region that contain a calcineurin binding site, but N-terminal regions vary and may confer different functions specific to the different isoforms. The RCAN1-1 promoter responds to glucocorticoids, but the RCAN1-4 promoter does not, and the two promoters are differentially activated in different tissues (Sun et al., 2011a).

Basal levels of RCAN1-1 are higher than RCAN1-4 in most tissues, including the brain (Ermak and Davies, 2013; Wu and Song, 2013). Expression of RCAN1-4 is strongly induced by increases in intracellular Ca^{2+} levels in response to stress, such as ischemia/reperfusion (I/R) injury or oxidative imbalance, via a calcineurin responsive promoter; this promoter contains a cluster of NFAT binding sites located on an intron immediately upstream of exon 4 (Sobrado et al., 2012; Wu et al., 2007). The CCAAT/enhancer-binding protein β (C/EBPβ) also cooperates with NFAT in the activation of RCAN1-4 transcription; C/EBPβ directly interfaces with NFAT, concurrently binding to multiple sites on the RCAN1-4 proximal promoter (Oh et al., 2010). Expression of RCAN1-4 is also activated by NF-κB, which binds to an NF-κB responsive site in the RCAN1-4 promoter independently of the calcineurin-NFAT pathway (Zheng et al., 2014). Both RCAN1-1 and RCAN1-4 inhibit NF-κB activity and nuclear translocation by depressing IκBα phosphorylation, and both were shown to inhibit the growth of human malignant glioma cells and lymphoma xenografts in mice (Chen et al., 2017; Zheng et al., 2014). Conversely, RCAN1-1 expression is less responsive to stress conditions, although its translational activation has been shown to transiently increase. However, RCAN1-1 protein levels, particularly those of RCAN1-1L, are elevated in Alzheimer's disease and can be induced by chronic stress, such as long-term exposure to glucocorticoids, that can induce amyloid β pathology (Hirakawa et al., 2009; Lupien et al., 1998; Sun et al., 2011a; U et al., 2004). It has been proposed that transient increases in RCAN1-1 and RCAN1-4 protein levels during long-term stress, lasting up to several hours, could be a protective stress adaptation. However, chronic elevations of RCAN1-1L have been implicated in the harmful effects of neurodegenerative diseases (Ermak and Davies, 2013). Selective upregulation of RCAN1-1L could be mediated by transcription factor activity, as transcription enhancer 3 (TEF3), alone among TEF family members, is required for RCAN1-1L expression in human endothelial cells responding to VEGF-A-induced angiogenesis, through specific binding to M-CAT elements in its promoter (Liu et al., 2008).

RCAN1: Both low AND high levels are inadequate

The partial monosomy 21 containing RCAN1 case referenced earlier exhibited a broad spectrum of symptoms associated with DS, suggesting that both overexpression and underexpression of RCAN1 can contribute to defective DS phenotypes. DS-associated genes exhibiting haploinsufficiency tend to be dosage sensitive (Antonarakis, 2017; Lyle et al., 2009). In support of this notion, RCAN1 knockout (KO) mice present with impaired hippocampal neurogenesis, reduced dendritic spine size and density, atypical axonal development, and mitochondrial dysregulation (Choi et al., 2019; Hoeffer et al., 2007; Min et al., 2012; Parra et al., 2018; Wang et al., 2012).

The efficacy of low RCAN expression is also reported in invertebrate and fungal models with only one copy of the RCAN gene (Table 2). RCAN-null mutants of an opportunistic pathogenic fungus, *C. neoformans*, show reduced virulence due to slow growth and failure of hyphal elongation (Fox and Heitman, 2005; Görlach et al., 2000). RCAN-null mutants of *C. elegans* present with defective thermotaxis and abnormal sensory behavior, and those overexpressing RCAN present with small body size, developmental delays, fertility defects, and abnormal behavior, suggesting nervous system defects (Lee et al., 2003; Li et al., 2015). Interestingly, RCAN duplicates accompanying complex genomic arrangement in *C.elegans* result in reduced expression and are linked to increased fitness and exploratory behavior (Zhao et al., 2020). In *Drosophila*, both overexpression and underexpression of RCAN are associated with severe learning defects and mitochondrial dysfunction (Chang and Min, 2005; Chang et al., 2003). RCAN overexpression delays neurodegeneration and protects against axonal transport defects caused by ectopic expression of amyloid precursor protein by restoring calcineurin and GSK3b signaling in fly eyes (Shaw and Chang, 2013; Shaw et al., 2015). Underexpression of RCAN in flies also causes defective ovulation with impaired meiosis and atypical courtship behavior (Ejima et al., 2004; Takeo et al., 2006). The study using yeast null mutants of RCAN indicates that the lack of RCAN can cause reduced calcineurin activity (Kingsbury and Cunningham, 2000).

RCAN1-4 protein content is increased in human muscles deficient in α-actinin 3 (ACTN3), a critical component of the Z-disk in fast-twitch skeletal muscle fibers (Papadimitriou et al., 2019). Approximately 16% of the global population is homozygous for a nonsense ACTN3 allele, and the loss of α-actinin-3 is associated with reduced power but enhanced endurance capacity associated with suboptimal metabolic and physiological fast-twitch fiber performance (Macarthur et al., 2007; 2008; North et al., 1999). RCAN1-4 is strongly induced in skeletal muscle in ACTN3 KO mice immediately after endurance exercise (Seto et al., 2013). Calcineurin promotes the transition from fast- to slow-twitch muscle activity in response to sustained, low-amplitude calcium production (Dolmetsch et al., 1997). α-actinin-3 deficiency results in increased calcineurin activity in skeletal muscle because of the binding of α-actinin-2 to calsarcin-2, a calcineurin inhibitor specific to fast-twitch muscle (Seto et al., 2013). α-actinin-2 is upregulated in ACTN3 deficiency, presumably through homeostatic compensation mechanisms (Papadimitriou et al., 2019; Seto et al., 2013). The expression of RCAN1-4 is increased by calcineurin under control of a cluster of 15 NFAT binding sites located in the RCAN1-4 promoter region, and

upregulated RCAN1-4 undergoes a negative feedback loop to inhibit further calcineurin activity (Rothermel et al., 2003). Therefore, RCAN1-4 regulates the adaptive development of muscle, which transforms in between fast- and slow-twitch fibers, responding to high contraction activity.

RCAN1-4 is translated via conventional cap-dependent initiation and cap-independent pathways in the soma and axons of hippocampal neurons (Seo et al., 2019). Death-associated protein 5 (DAP5) enhances cap-independent translation by binding to a *cis* regulatory element located on the 5′ untranslated region (UTR) of the RCAN1-4 transcript. Brain-derived neurotrophic factor (BDNF), known to support neural differentiation and survival, induces DAP5 expression, and the resultant axonal RCAN1-4 translation enhancement is critical to axon outgrowth.

RCAN1 in organelles

Non-cytoplasmic RCAN1 has also been reported in subcellular organelles, including the nuclei and mitochondria of fruit flies and mammals (Chang and Min, 2005; Sun et al., 2011a).

Nucleus: RCAN1 has been detected neuron nuclei in mammals and fruit flies (Chang and Min, 2005; Leahy and Crawford, 2000; Parra et al., 2018; Pfister et al., 2002; Rothermel et al., 2000; Sun et al., 2011a). Nuclear localization of RCAN1 may depend on the interaction with calcineurin; calcineurin is shown to translocate into the nucleus when activated by a replaced autoinhibitory domain. The C-terminal region of RCAN1 is reported to be critical for nuclear localization and calcineurin interaction (Pfister et al., 2002). Apoptosis of neuroprogenitor cells induced by overexpression of RCAN1 is suppressed by zinc treatment, which facilitates the formation of RCAN1 aggregates in the nucleus (Lee et al., 2007).

RCAN1-1S has been reported to interact with histone deacetylase 3 (HDAC3) via its N-terminal region (Han et al., 2014). In cultured cells, exogenously expressed RCAN1-1S is acetylated, and HDAC3 stabilizes RCAN1-1S by inhibiting ubiquitination due to deacetylation. HDAC3 also promotes the nuclear translocation of RCAN1-1S.

RCAN1 contains an RNA binding domain and was recently shown to bind to specific RNAs to help process or stabilize them (Strippoli et al., 2000). Specifically, the RCAN1-1S protein directly binds to adenine nucleotide translocator 1 (ANT1) mRNA at a region comprising the first 327 nucleotides, purportedly to stabilize the transcripts; this, in turn, leads to mitochondrial dysfunction (Jiang et al., 2017; Yun et al., 2019). The putative RNA binding domain is located at the N-terminal, 1 to 103 amino acid region of RCAN1-1S, a potential therapeutic target for the neuroprotective RNA aptamer that blocks pathological RCAN1 activity.

RCAN1 also binds to ten–eleven translocation 1 (TET1) introns to negatively regulate the splicing process by interfering with the binding of spliceosome machinery to TET1 transcripts (Choi et al., 2019). As such, increased TET1 activity facilitates the demethylation of the miRNA124 promoter, thereby increasing transcription thereof in RCAN1 KO mice; conversely, decreased TET1 activity reduces this transcription of miRNA124 in mice overexpressing RCAN1. This dysregulated expression of miRNA124 results in memory deficits because of defective adult hippocampal neurogenesis.

Mitochondria: RCAN1 has been shown to help maintain mitochondrial structural and functional integrity. In *Drosophila*, overexpression or underexpression of RCAN1 causes severe defects in learning and memory associated with mitochondrial instability (Chang and Min, 2005). In flies overexpressing or lacking RCAN1, ATP levels drop while ROS levels increase. The mitochondrial DNA content decreases in those flies, while the number of mitochondria increases, but their average size decreases, suggesting that mitochondrial fission seems to override fusion events. The mitochondrial phenotypes observed in flies overexpressing or lacking RCAN1 are not rescued by reduction of calcineurin activity, suggesting that RCAN1's function on mitochondria may not be solely dependent on calcineurin.

Mitochondrial DNA content is also significantly decreased in human T21 fetal brain tissue, and catalytic inefficiency of mitochondrial complex 1 is observed in DS human fetal fibroblasts (Valenti et al., 2011). Drastic decreases in cAMP levels due to low protein kinase A (PKA) activity diminish cAMP-dependent phosphorylation of the 18 kDa subunit of complex 1, which, in turn, leads to overproduction of ROS. PKA phosphorylates RCAN1 to stabilize (Kim et al., 2012). Therefore, low activity of PKA in DS cells is likely to be compensatory to presumably enhanced expression of RCAN1.

Overexpression of RCAN1-1 has also shown pro-apoptotic activity in mammalian primary neurons through activation of caspase-3/7 and caspase-9, (though not caspase-11) facilitated by the release of cytochrome c from mitochondria into the cytosol (Sun et al., 2011a). Particularly, overexpression of RCAN1-1S increases both mRNA and protein levels of ANT1 by stabilizing *ANT1* mRNA in primary neurons and in human neuroblastoma cell lines (Jiang et al., 2017). ANT1 accumulation accelerates the ATP–ADP exchange rate, promoting the opening of mitochondrial permeability transition pores (mPTPs) and subsequent cytochrome c release; these lead to mitochondrial swelling associated with eventual cell death. The upregulation of ANT1 by RCAN1-1S occurs independently of calcineurin activity, and calcineurin blockers do not affect ANT1 levels. Conversely, overexpression of RCAN1-1L was shown to inhibit hypoxia-induced cell apoptosis in human umbilical vein endothelial cells (HUVECs) and human adult cardiac myocytes, whereas knockdown of RCAN1-1L accelerated it (Sun et al., 2014). Overexpression of RCAN1-1L is reported to induce mitophagy, activating autophagy flux, translocation of the mitophagy receptor Parkin to mitochondria, and opening of mPTPs. The upregulation of RCAN1-1L may thereby contribute to cell survival under hypoxic conditions.

Conversely, cardiomyocytes in RCAN1 KO mice and RCAN1-1-depleted mammalian cardiomyocytes show increased mitochondrial fragmentation (Parra et al., 2018). RCAN1 depletion translocates DRP1 to the mitochondria to induce fission, and cardiomyocytes harboring fragmented mitochondria are more susceptible to I/R insults because of increased activity of calpain, a Ca^{2+}-activated cell-damaging protease, resulting from impaired low Ca^{2+}-buffering capacity therein. Interestingly, mitochondria fusion is increased in induced pluripotent stem cells (iPSs) derived from human DS individuals, indicating that RCAN1 is critical to mitochondrial

network maintenance in cardiomyocytes.

Diabetes is frequently associated with DS (Johnson et al., 2019). DS β-cells exhibit fragmented mitochondria associated with reduced energy metabolism and secrete high levels of proinsulin, indicating incomplete insulin processing and secretion (Helguera et al., 2013). A recent syntenic, cross-species aneuploidy genetic screen identified the RCAN1 gene as a link between hyperglycemia and dysfunctional phenotypes associated with diabetes in DS β-cells (Peiris et al., 2016). RCAN1 was the only upregulated gene in a comprehensive gene expression analysis of human diabetic β-cells among genes in a region of chromosome 21 associated with hyperglycemia in mice models. RCAN1 is upregulated in diabetic β-cells in response to high glucose exposure, and mice overexpressing RCAN1 presented with inhibited glucose-stimulated insulin secretion and mitochondrial dysfunction in their β-cells (Peiris et al., 2012). Because insulin gene transcription is regulated by the calcineurin/NFAT signaling pathway, long-term upregulation of RCAN1 in β-cells likely suppresses insulin gene expression (Heit, 2007).

RCAN1 modulation of circadian rhythms

Significant circadian oscillations of metabolism and gene expression are observed in many different organs, including cardiovascular tissues (Bray and Young, 2008; Tsai and Young, 2009). In mouse hearts, RCAN1-4 protein and transcript levels fluctuate throughout the day, from low in the morning to high in the evening (Bray et al., 2008; Sachan et al., 2011). This cycle may diminish calcineurin activity, reducing phosphorylation of heart contraction proteins, as these nocturnal animals reduce their activity towards the end of the dark cycle. However, circadian fluctuation of RCAN1 expression was not shown to contribute to diurnal corticosterone production, as evidenced by unchanged fecal corticosterone levels in RCAN1-knockout mice and their wildtype littermates (Rakowski-Anderson et al., 2012). In murine models and humans, cardiac damage from I/R is at its highest during the transition from sleeping to waking, when RCAN1 expression is at its lowest (Durgan et al., 2010; Martino and Sole, 2009). Wildtype mice infarcts are at their most frequent and severe after I/R surgeries performed during the transition from day to night (Rotter et al., 2014). Regardless of surgery timing, RCAN1 KO mice consistently show the greatest damage, whereas overexpression of RCAN1 in transgenic mouse cardiomyocytes was shown to be cardioprotective after I/R surgery. Many studies have shown that cardiovascular events in humans are most likely in the morning, during the transition from rest to activity (Manfredini et al., 2013; Martino and Sole, 2009). Taken together, such findings suggest circadian fluctuation of the RCAN1/calcineurin signaling pathway is involved in cardiovascular sustainability.

RCAN1 in thermogenesis and obesity

Thermogenesis regulates energy expenditure by generating heat and reducing fat storage of excess calories, and the disruption of thermogenic homeostasis has been associated with obesity and metabolic disease (Bouret et al., 2015). A recent report suggested that RCAN1 suppressed thermogenesis, thereby contributing to the control of energy expenditure (Rotter et al., 2018). RCAN1 KO mice are resistant to high-fat diet-induced obesity because of an increase in whole-body metabolic rate following upregulation of thermogenesis. Expression levels of mitochondrial uncoupling protein 1 (Ucp1) in subcutaneous white adipose tissue (WAT) and sarcolipin in skeletal muscle are increased in RCAN1 KO mice, leading to upregulated heat production and whole-body metabolism and resultant reduced lipid accumulation.

Thermogenic regulation by RCAN1 might be dependent on calcineurin because transcription of Ucp1 and sarcolipin corresponds to calcineurin activity (Rotter et al., 2018). Consistent with these observations, organ transplant patients receiving calcineurin inhibitors to manage graft rejection showed an increased risk for weight gain and new-onset diabetes (Heisel et al., 2004; López-Vilella et al., 2015). Human genome-wide association studies indicate that the locus encoding the calcineurin A subunit is associated with body mass index and insulin levels (Ramos et al., 2014). Moreover, a polymorphism in *Rcan1* is associated with an increased risk of colorectal cancer, for which obesity is an established risk factor (Sebio et al., 2015). Another report showed that the loss of RCAN1 increased mitochondrial fission calcineurin-dependently and was associated with the white-to-beige conversion of human adipocytes facilitated by Upc1 expression in WAT (Pisani et al., 2018; Rotter et al., 2018).

In regions colder than normal laboratory culture conditions, *rcan-1* loss-of-function mutants of *C. elegans* were cryophilic, as were calcineurin gain-of-function mutants wherein calcineurin is constitutively active (Li et al., 2015). Also, *crtc-1* is a substrate of calcineurin and regulates mitochondrial metabolism and lifespan (Burkewitz et al., 2015; Mair et al., 2011). RCAN1 may thus have been involved in adaptive thermogenesis responses via multiple distinct but related mechanisms throughout evolution.

RCAN1 is also implicated in obesity induced by impaired estrogen signaling (Ribas et al., 2016). The incidence of insulin-resistant type II diabetes is lower in premenopausal women and men, but incidences of this and other chronic metabolic diseases, including obesity and certain types of cancers, increase significantly for women after menopause (Moley and Colditz, 2016). Both RCAN1-1 and RCAN1-4 were upregulated in mice lacking estrogen receptor α (ERα), specifically in skeletal muscle, the primary insulin-responding tissue that conducts oxidative metabolism of absorbed glucose (Ribas et al., 2016). In skeletal muscle with impaired ERα, aberrant mitochondrial morphology and mitochondrial DNA turnover were diminished, and ROS levels were elevated. Mitochondrial fission-and-fusion dynamics are dependent on Drp1, and impaired mitophagic signaling and autophagy are influenced by calcineurin. Overexpression of RCAN1 in primary myotubes impairs insulin potency. These findings indicate that the RCAN1-calcineurin axis may be a critical regulatory point in estrogen-driven insulin responses, through proper maintenance of mitochondria.

RCAN1 in the kidney

RCAN1 is reported to be suppressed in glomeruli in many different types of human nephropathy. The RCAN1 locus is hypermethylated, and RNA expression of RCAN1 is down-

regulated in human primary podocytes infected with HIV-1 (Li et al., 2018). Pretreatment with a DNA methyltransferase inhibitor rescues low expression of RCAN1 to homeostatic levels in cultured podocytes infected with HIV or bathed in concentrated glucose. Loss of the RCAN1 gene aggravates albuminuria and podocyte injury in mice with nephropathy. Epigenetic suppression of RCAN1 expression thus contributes to podocyte injury in the contexts of HIV infection and hyperglycemia.

Serum levels of fibroblast growth factor 23 (FGF23) are highly elevated in chronic kidney disease (CKD); this activates FGFR4 in cardiac myocytes to stimulate the calcineurin pathway alongside the upregulation of RCAN1, RCAN4, and NFAT (Grabner et al., 2015). Enhanced FGF23 signaling in CKD contributes to the development of left ventricular hypertrophy during mineral metabolism alterations, such as hyperphosphatemia and hypercalcemia (Leifheit-Nestler et al., 2018; Liu et al., 2018).

RCAN2: Cancer, ciliogenesis, and skeletal growth

RCAN2 (previously ZAKI-4) maps onto human chromosome 6 (Cao et al., 2002). RCAN2-1 and RCAN2-2 transcripts encode for the same protein, referred to as RCAN2-3 or RCAN2L (previously RCAN2β) and consist of 243 amino acids. The RCAN2-4 transcript produces a protein called RCAN2-4 or RCAN2S (previously RCAN2α) consisting of 197 amino acids (Serrano-Candelas et al., 2014).

Recent reports have expanded our understanding of the different functions of RCAN2. RCAN2 is broadly expressed in many tissues, including the brain, heart, striated muscle, liver, and bone (Bassett et al., 2012; Sun et al., 2011b). RCAN2-positive human gastric cancers are more advanced than RCAN2-negative ones, and the overexpression of RCAN2 accelerates cell growth and enhances gastric cancer invasiveness in cell lines while RCAN2 knockdown decreases it (Hattori et al., 2019). Conversely, RCAN2 tends to be downregulated in KRAS-mutated colorectal cancer, such that calcineurin-NFAT signaling is derepressed to allow the proliferation of tumor cells (Hattori et al., 2019; Niitsu et al., 2016).

RCAN2 is localized to the centriole and controls ciliogenesis in zebrafish and human cells; RCAN2 was upregulated upon the loss of giantin, a Golgi matrix protein required for ciliogenesis, that is regulated by calcineurin activity (Hu et al., 2006; Stevenson et al., 2018).

Mice lacking RCAN2 display delayed intramembranous ossification and reduced bone mineral accumulation during development and over-mineralization in adult bone (Bassett et al., 2012). Researchers also found that RCAN2S expression levels in bone were regulated by thyroid status. The RCAN2 knockout mice presented with reduced food intake and attenuated hyperphagic responses to starvation, slowing growth and suppressing age- or diet-induced obesity through leptin-independent mechanisms (Sun et al., 2011b). Unlike thermogenic RCAN1 suppression in WAT and skeletal muscle, the major site of RCAN2 action was apparently in or around the hypothalamus, which controls appetite and food consumption.

RCAN3: Constitutive expression

RCAN3 is located on chromosome 1. Although more than 10 different transcript variants have been reported, only one RCAN3 protein (previously known as DSCR1L2) consisting of 241 amino acids is produced from transcripts containing exons 4, 5, 6, and 7 (Facchin et al., 2011; Serrano-Candelas et al., 2014; Strippoli et al., 2000). RCAN3 is reported to be a phosphoprotein that interacts with human cardiac troponin I (TNNI3), RAF kinases, and calcineurin (Canaider et al., 2006; Facchin et al., 2008; Martínez-Høyer et al., 2013; Mulero et al., 2007; Serrano-Candelas et al., 2015). RCAN3 inhibits the proliferation of HUVECs, and higher constitutive expression of RCAN3 than inducible expression of RCAN1 underscores the physiological relevance of RCAN3 (Canaider et al., 2010). Overexpression of RCAN3 was shown to modulate T cell development by increasing positive selection and suppressing pro-inflammatory T cell differentiation in cell culture and in arthritis development induced by collagen injection in murine models (Park et al., 2017; Serrano-Candelas et al., 2015). RCAN3 overexpression also inhibited tumor growth and angiogenesis calcineurin-dependently in an orthotopic human breast cancer model, and the expression of an RCAN3-derived peptide containing the calcineurin-binding motif reproduced these anti-tumor effects (Martínez-Høyer et al., 2015). A recently discovered single nucleotide polymorphism in RCAN3 was reportedly associated with reading and spelling capacity and awaits confirmation (Meijsen et al., 2019).

CONCLUSION REMARKS

RCAN was first identified as DSCR1 and then discovered to be an important endogenous RCAN, making it critical to multiple cellular processes. Although RCAN was initially implicated in the pathology of DS, unprecedented and unexpected functions of RCAN family proteins continue to emerge because of sweeping advancements in biotechnology and bioinformatics, the availability of genome-wide big data of increasing numbers of DS cases, and the increasing utilization of model systems. Three different RCAN isoforms have been found to be expressed in many different tissues and encompass a wide range of molecular functions. These isoforms are predominantly found in the cytoplasm but also localize to nuclei and mitochondria. Findings indicate that RCAN functions involve RNA binding, mitochondria regulation, circadian rhythm modulation, thermogenesis, and metabolism, offering favorable potential for RCAN as a therapeutic target. As these functions surpass those anticipated by its original implication in DS pathology, future research is expected to explore and expand on these functions, with more expected to emerge.

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S.K.L. and J.A. wrote the manuscript and secured funding.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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