Chapter 10

RCAN1 and Its Potential Contribution to the Down Syndrome Phenotype

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1. Introduction

Down Syndrome (DS) is caused by trisomy of *Hsa21* in humans [1]. It is the most common autosomal aneuploidy, occurring in about 1 in 700 live births [2]. The clinical features of DS are variable and affect many different aspects of development. In any given individual, there may be over 80 different clinical traits [3]. Major clinical features associated with DS include the distinctive craniofacial appearance, reduced size and altered morphology of the brain, cognitive impairments, hearing loss and defects of the gastrointestinal, immune and endocrine systems [3]. Whilst this constellation of anomalies has been described we are still far from understanding their cause. How does an extra set of normal *Hsa21* genes result in whole body system disturbances and what are the molecular genetics bases for these disturbances?

A large number of genes are simultaneously expressed at abnormal levels in DS, therefore, it is a challenge to determine which genes contribute to specific abnormalities, and then identify the key molecular pathways involved. We are advocates of the approach articulated by Nadel [4] - that a careful and detailed analysis of the clinical defects in humans be followed by the creation of mouse models that over-express only some of the genes triplicated on *Hsa21*, so that the genes responsible for specific features of the DS phenotype can be identified. We generated mice in which the *RCAN1* gene is over-expressed (RCAN1-TG) to study the consequences of excess RCAN1 and thus investigate its potential contribution to the DS phenotype. Our research adds to the growing body of work assigning specific functions to particular *Hsa21* genes. Other examples under study with a particular focus on brain function include, *DYRK1A* [5], *SOD1* [6], *APP* [7] [8] [9], *SNYJ1* [10] and *ITSN1* [11]. Once we understand the abnormalities caused by subtle over-expression of single genes, we can embark on a programme to generate mice expressing combinations of genes to examine potential additive effects. This sort of approach is consistent with the idea that the DS phenotype results from disturbances in biological path-

ways due to an accumulation of subtle changes brought about by the effects of the over-expression of many single genes. Indeed, such an approach is bearing fruit already - *RCAN1* and *DYRK1A* have been shown to act cooperatively to destabilise a calcineurin regulatory circuit when the genes are over-expressed in a combinatorial fashion [12].

The focus of this chapter will be to provide insight into *RCAN1* and its functions, and examine the evidence to suggest that this gene plays a role in the neurological, immune and vascular systems. We will firstly give an overview of the gene family to which *RCAN1* belongs; followed by a description of the functional domains of the protein product, including post translational modification domains; its tissue expression pattern; cellular pathways involving RCAN1; and finally, how its over-expression may contribute to the neurological, immune and cancer phenotypes associated with DS.

2. The RCAN gene family

DSCR1, renamed *RCAN1,* was first described by our group in 1995 after a search for genes located on *Hsa21* with the potential to be involved in DS [13]. *RCAN1* is a member of a family of calcineurin binding proteins and is conserved across species, from lower unicellular eukaryotes such as yeast to complex organisms including humans [14]. The high level of interspecies homology of this protein has been taken to indicate a conserved role during evolution [15] [16]. A number of different genes belonging to this family have now been identified in humans, including, *RCAN1, RCAN1L2, RCAN2* and *RCAN3* [15, 17]. The family was identified based on the presence of a short "signature" polypeptide FLISPPxSPP (part of the so called SP motif) [18] but there is a high degree of similarity across the entire protein in all RCAN family members. All members perform similar functions. For example, RCAN2 interacts with calcineurin with similar efficiency to RCAN1 [19] and the human gene can functionally replace the yeast gene [18]. Interestingly, while RCAN family members are all expressed in similar tissues, each family member displays a distinct expression profile. For instance, while all family members were expressed in the brain, each displayed different levels of expression, depending on the region and developmental stage examined [20]. Within these regions there were also differences in the cellular and subcellular location of the family members. RCAN1 was highly expressed in neurones and in the neutropil, while RCAN1L2 was expressed in scattered neurones and was the only RCAN family member detected in glial cells [20, 21]. The differential expression pattern of the RCAN family members in the brain indicates that they are all likely to be important in brain development and function, yet each member may be functionally distinct [20].

3. General tissue and cellular expression of RCAN1

The *RCAN1* gene spans about 100 kb of genomic DNA and consists of seven exons and six introns. Of the seven exons, the first four are alternative first exons (*RCAN1-1* to *RCAN1-4* containing exons 1 to 4, respectively). *RCAN1* encodes two major protein isoforms, RCAN1-1

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and RCAN1-4. RCAN1-1 protein consists of 252 amino acids, while RCAN1-4 is a shorter, 197 amino acid protein [22, 23]. Using Northern blot analysis, *RCAN1-1* and *RCAN1-4* were found to be similarly distributed throughout the body [22]. *RCAN1-1* was highly expressed in the foetal brain and in the adult brain, heart and skeletal muscle. Lower levels were detected in the foetal lung, liver and kidney and in the adult pancreas, lung, liver and placenta. High levels of *RCAN1-4* were detected in the foetal kidney and in adult heart, skeletal muscle and placental tissues, with lower levels in the foetal brain, lung and liver and adult lung, liver, kidney and pancreas. While both isoforms exhibited a similar expression pattern, only very low levels of *RCAN1-4* were found in the adult brain and *RCAN1-1* expression could not be detected in the adult kidney [13, 22]. Northern blot and RT-PCR failed to detect exon 3 in any of the foetal or adult tissue studied, while isoform 2 was found only in the foetal brain and liver [22].

RCAN1-1 and RCAN1-4, the most predominantly expressed isoforms, are under the control of different promoters and are therefore likely to have different regulatory mechanisms and possibly even different functions. For example, *RCAN1-4* expression is regulated by calcium signalling. Experiments in PC-12 cells (a neuronal like cell line) found that when intracellular calcium levels increased through membrane depolarisation, *RCAN1-4* gene expression was rapidly induced [24] and this was mediated by the calcineurin/Nuclear factor activated T cells (NFAT) signalling pathway [24]. Studies on the *RCAN1-4* promoter identified the presence of putative NFAT binding sites. No study published to date has demonstrated Ca^{2+}/calcineurin-mediated expression of *RCAN1-1*. Interestingly, RCAN1 is able to function in an autoinhibitory manner as over-expression of any RCAN1 isoform resulted in an inhibition of *RCAN1-4* gene expression [24].

The subcellular location of RCAN1 protein was initially determined using tranfection of a RCAN1-GFP protein construct in C2C12 cells, a mouse myoblast cell line. RCAN1 protein was located in both the nuclear and cytosolic compartments and in the absence of treatments to activate the calcineurin signalling pathway, resided predominantly in the nucleus [25]. Various physiological and biochemical stresses have been demonstrated to influence the location of RCAN1 within a cell. For example, under normal circumstances RCAN1 was located within the nuclear compartment in various cell lines, including HT-1080 fibrosarcoma and I251 astroglioma cells. However, when these cells were subjected to oxidative stress, RCAN1 protein was redistributed to the cytoplasm [26]. The same observation was made following activation of the calcineurin signalling pathway, which resulted in the translocation of RCAN1 from the nucleus into the cytosolic compartment [27].

4. Functional domains of the RCAN1 protein

Initial studies found that both RCAN1 isoforms encode a proline rich protein consisting of a putative acidic domain, a serine proline motif, a putative DNA binding domain and a proline rich region typical of a SH3 domain ligand [22, 28]. These structural motifs are typically seen in proteins involved in transcriptional regulation and signal transduction. A more recent study on RCAN1 proteins in dozens of species revealed 4 highly conserved regions separated by

other regions that are less well conserved. These four regions consist of: a region at the amino terminus capable of forming an RNA recognition motif; the gene family signature domain consisting of the highly conserved SP motif; a PxIxIT-like domain (x represents any amino acid) and a C-terminal TxxP motif [29] (see Figure 1). The functions of these highly conserved regions in RCAN1 proteins are yet to be fully explored.

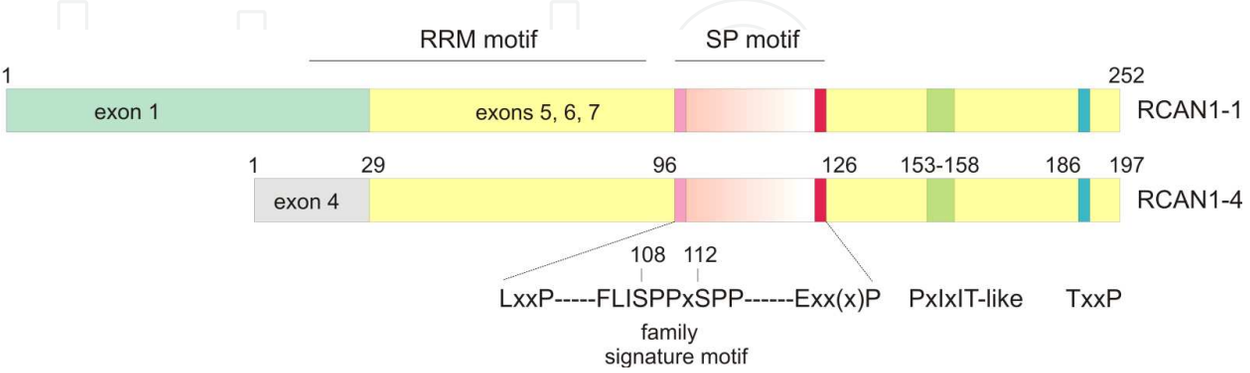

Figure 1. Schematic representation of the major RCAN1 protein isoforms. Protein motifs are shown: the RRM (RNA recognition motif); the SP (serine / proline) motif incorporating the LxxP, family signature and ExxP domains; the PxIxIT-like domain; and the TxxP motif. Serines 108 and 112 in RCAN1-4 are also indicated.

The most highly conserved region in the RCAN1 protein is the SP motif. This motif is similar to that present in NFAT proteins [30]. *In vitro,* the SP motif is able to bind to and inhibit calcineurin activity, however studies in cell lines have suggested that it is not necessary or sufficient to achieve this. By generating various deletion-constructs of the RCAN1 coding sequence it was found that RCAN1 was able to inhibit calcineurin in C2C12 myoblasts even when the SP domain was absent [31]. This study determined that two additional domains, one at the N-terminus, the other in the distal C-terminal region, were required to inhibit calcineurin activity [31]. Use of a truncated version of the RCAN1 protein also demonstrated that the last 33 amino acids were essential for nuclear localisation. In the absence of this 33 amino acid domain (which contains the SP motif and a region identified as a SH2 domain) RCAN1 protein accumulated in the cytoplasm [25].

Site-directed mutagenesis studies have shown that phosphorylation of the RCAN1 protein regulates its function, subcellular location and stability. Indeed, RCAN1 can be phosphorylated by various kinases at a number of different sites to change its activity towards calcineurin. For example, the serine residue within the SP domain at position 112 (Ser^{112}) (Ser^{167} in RCAN1-1) is variously phosphorylated by BMK1 [32], NIK [33] and DYRK1 [34] and acts as a priming site for subsequent phosphorylation at Ser^{108} (Ser^{163} in RCAN1-1) by GSK-3 [35] [31] [34]. Phosphorylation by TAK1 at Ser^{94} and Ser^{136} [36] and by DYRK1A at Thr^{193} [34] also change the activity of RCAN1 towards calcineurin (see later). NIK-mediated phosphorylation [33] or phosphorylation by PKA [37] augmented the half-life of RCAN1 protein. And, phosphorylation of a threonine residue (Thr^{166} in RCAN1-4) in the SH2 domain controlled its subcellular localisation since exchanging the threonine for an alanine resulted in an accumulation of RCAN1 protein within the cytoplasm [25]. Thus, nuclear localisation of RCAN1 is controlled, at least in part, by phosphorylation.

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Other studies have shown that RCAN1 is cleaved by calpain and this cleavage appears to increase the stability of the protein by decreasing its proteasome-dependent degradation [38]. Further, the cleavage of RCAN1 by calpain also affects its interactions with other proteins. For example, cleavage of RCAN1-4 by calpain abolished its ability to bind to Raf-1 [38]. Yet another pathway involved in the post translational regulation of RCAN1 is the ubiquitin-proteasome system (UPS). The UPS is important in the regulation of protein turnover in response to changing cellular conditions and facilitates the degradation of defective proteins [39]. Ubiquitin is a polypeptide able to bind to lysine residues on proteins targeted for degradation. This binding occurs through sequential steps mediated by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) [40]. Following this sequence of events, the 26s proteasome is able to recognise and degrade the poly-ubiquinated protein. The first evidence to suggest that RCAN1 was degraded by the ubiquitin pathway came from yeast two hybrid and co-immunoprecipitation experiments which found that RCAN1-4 interacted with ubiquitin [41]. More recent studies demonstrated that RCAN1 interacts with other members of the UPS, including, Skp1, Cullin/Cdc53, F-box protein Cdc4 (SCF^{Cdc4}) [42] and $SCF^{\beta\ TrCP1/2}$ [40]. The interaction between RCAN1 and the UPS is not only important in regulating turnover of the RCAN1 protein but may also influence its function. For example, increased degradation of RCAN1 by SCF^{Cdc4} diminished its ability to inhibit calcineurin signalling [42].

5. RCAN1 function—Signal transduction pathways

Interest in *RCAN1* surged after the discovery that it encoded a protein capable of inhibiting the protein serine/threonine phosphatase calcineurin (PP2B/PPP3C) [19] [27] [31] [43] [44]. RCAN1 has since been implicated in a variety of cellular processes, including oxidative stress [45] [46] [47] [48], angiogenesis [49], mitochondrial function [50] and immune responses and inflammation [44] [51]. Participation of RCAN1 in these processes has been mostly attributed to its interaction with the calcineurin pathway. Nonetheless, calcineurin-independent activities have been demonstrated [51] [52] [53] [54] [55]. Recently, RCAN1 mRNA and protein was found to increase in the peri-infarct region following middle cerebral artery occlusion (MCAO) in mice [56] and its up regulation was found to be protective [57].

5.1. The calcineurin pathway

The calcineurin pathway plays an integral role in the development and homeostatic regulation of a number of different cell types, including immune cells and neurones. The pathway is activated by increases in intracellular calcium (Ca^{2+}) due to oxidative stresses, chemical-mediated calcium increases and in response to biomechanical strain [58]. An increase in intracellular Ca^{2+} leads to the activation of calmodulin, which forms a complex with calcineurin to activate its phosphatase function. Activated calcineurin then dephosphorylates cytosolic NFAT leading to its translocation to the nucleus where it complexes with GATA-4 [59] allowing DNA binding and facilitation of the transcription of numerous gene targets [60].

RCAN1 interacts directly with calcineurin [19] [27]. Calcineurin is a heterodimer, consisting of a catalytic A subunit and a calcium binding regulatory B subunit [61]. RCAN1 is able to bind to the A subunit in a linker region between the calcineurin A catalytic domain and the calcineurin B binding region [19]. Deletion of the carboxyl-terminal half of the catalytic domain of calcineurin A abolished binding with RCAN1, indicating that this region was critical for the interaction [27]. Studies with RCAN1 have shown that exon 7 is able to bind to and regulate the activity of calcineurin and this binding occurs with a very high affinity [62]. While binding of RCAN1 to calcineurin did not interfere with the interaction between calcineurin and calmodulin, it is believed to interfere with the ability of calcineurin to bind NFAT by competing with the NFAT binding site [31]. Indeed, when *RCAN1* was over-expressed, it inhibited the activity of an exogenously added constitutively active calcineurin and transcription of a number of calcineurin-dependent genes including *IL-2* and *MEF2* was prevented [27]. *RCAN1* over-expression was found to inhibit NFAT translocation to the nucleus, thus inhibiting calcineurin-dependent gene transcription [19] but was unable to inhibit a constitutively active form of NFAT demonstrating that the inhibition of calcineurin signalling was through calcineurin, rather than interference with downstream components of the pathway [27].

Interestingly, activation of calcineurin signalling induces *RCAN1-4* expression [18, 19]. This occurs through a 900 base pair sequence located between exons 3 and 4 in an intragenic promoter region for *RCAN1-4*, which contains a dense cluster of consensus binding sites for the NFAT transcription factor [61]. The existence of such a site suggested that RCAN1 participates in a negative feedback loop, presumed to exist to prevent the adverse effects of unrestrained calcineurin activity following prolonged Ca^{2+} stimulation [27]. Indeed, following induction of the calcineurin pathway, levels of *RCAN1-4* mRNA increased within 1.5 hours and peaked 6 hours after treatment with a calcium stressor [45].

As more and more studies have emerged on RCAN1 and the propagation of calcium signals in the cell, it has become clear that the role of RCAN1 is not always to inhibit the calcineurin pathway. While the earliest studies found RCAN1 to negatively regulate the pathway, in other circumstances it seems to facilitate calcineurin activity. Indeed, contrary to expectations it was found that the absence of *Rcan1* diminished calcineurin signalling in yeast [18]. Similar results were found when *Rcan1* expression was disrupted in mice. *Rcan1*-null mice exhibited an unexpected decrease in calcineurin activity in the heart under normal physiological conditions and after stress [63] and a reduction of calcineurin activity was concomitant with reduced nuclear distribution of NFAT and a loss of NFAT-dependent gene transcription [64].

These apparently paradoxical actions of RCAN1 may be explained, at least in part, by its cellular concentration, its nuclear or cytosolic localisation and/or its phosphorylation status [64] [35] [32] [65] [25]. For example, the abundance of RCAN1 in the cell may determine its ability to either enhance or inhibit calcineurin signalling. Low or intermediate levels of RCAN1 were shown to facilitate calcineurin signalling while very high levels of over-expression were inhibitory, suggesting that RCAN1 oscillates between stimulatory and inhibitory forms depending on its concentration [35] [138]. In contrast, in another study, the functional role of RCAN1 was found to change in a dose-dependent fashion, but in the opposite direction to the

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aforementioned studies – RCAN1 was an inhibitor at low levels but a facilitator when levels were high [66]. Another study indicated that 4 highly conserved domains in the RCAN1 protein were important in determining its activity towards calcineurin. Specifically, that preferential binding of RCAN1 to calcineurin prevented NFAT binding resulting in inhibition of calcineurin signal transduction due to competition between RCAN1 and NFAT for calcineurin docking sites [29]. This preferential binding occurred in the presence of high levels of Rcan1 and required the LxxP domain within the SP motif and the PxIxIT domain [29]. Conversely, when Rcan1 was expressed at lower levels, the protein was able to stimulate calcineurin signalling. This stimulatory effect required the LxxP and ExxP domains within the SP motif as mutations within both of these domains prevented stimulation.

Other studies have suggested that it is the phosphorylation status of RCAN1 that determines its action as either an inhibitor or facilitator of calcineurin activity. A study in yeast found that for Rcan1 to facilitate calcineurin signalling it required phosphorylation of both serine residues located within the SP motif by a priming kinase (in this case MAPK) and Mck1, a member of the glycogen synthase kinase 3 (GSK-3) protein family. When the serines were mutated to alanines or in the absence of Mck1, Rcan1 was no longer able to stimulate calcineurin signalling resulting in inhibition [35]. Phosphorylation by TAK1, DYRK1A and NIK all switch RCAN1 from an inhibitor to a calcineurin facilitator [33] [32] [34]. At odds with most studies, phosphorylation of the serine residues within the SP motif of RCAN1 was reported to enhance its ability to inhibit calcineurin [23].

In summary, although the mechanisms responsible for the dual role of RCAN1 in the calcineurin signalling pathway is still under investigation, the results so far indicate that the primary function of RCAN1 is to facilitate calcineurin activity and this occurs when RCAN1 is expressed at lower or physiological levels. On the other hand, when RCAN1 is highly expressed, it has a secondary role of inhibiting calcineurin signalling by interfering with the interaction between calcineurin and NFAT.

5.2. GSK–3 signalling

Numerous studies outlined above have shown that GSK-3 phosphorylates RCAN1 to regulate its function. Interestingly, GSK-3 activity can also be regulated by RCAN1. PC-12 cells overexpressing RCAN1 displayed an increase in the absolute levels of GSK-3β protein, which in turn increased its kinase activity towards Tau [67]. Tau protein is a known target of GSK-3 which in its hyperphosphorylated form has been implicated in the aetiology of Alzheimer's disease [67]. Exactly how RCAN1 regulates the abundance of GSK-3 remains undetermined, but it seems that RCAN1 is acting at a post-transcriptional level as the amount of *GSK-3β* mRNA did not change upon increasing RCAN1 expression [67].

5.3. The MAPK/ERK signalling pathway

The MAPK/ERK signalling pathway mediates signal transduction from cell surface receptors to downstream transcription factors. This pathway plays a role in a number of cellular processes including proliferation, growth, motility, survival and apoptosis [68]. As indicated

above, MAPK was able to phosphorylate RCAN1 at S^{112} within the SP motif to prime its subsequent phosphorylation by GSK-3. Moreover, the same study demonstrated that phosphorylation of RCAN1 by MAPK allowed RCAN1 to become a substrate for calcineurin [31], thus introducing a further level of control to keep the pathway operating at an optimal level.

5.4. The NFκβ inflammatory pathway

RCAN1 is also able to regulate the Nuclear factor κβ (NFκB) signalling pathway. NFκB is a transcription factor that regulates target genes involved in many physiological processes, including immunity, inflammation, cancer, synaptic plasticity and memory. Under normal circumstances, NFκB exists as a dimer and is sequestered in the cytoplasm through its interaction with an inhibitory molecule known as Inhibitor of κB (IκB). Upon stimulation of the NFκB signalling pathway, IκB is degraded by the ubiquitin/proteasome pathway releasing its inhibitory action on NFκB [69]. Degradation of IκB allows NFκB to translocate to the nucleus where it acts to induce the expression of various target genes including the inflammatory genes cyclooxygenase-2 (*Cox-2*) and interleukin 1 (*IL-1*) [69]. RCAN1 is able to negatively regulate the NFκB signalling pathway by attenuating NFκB activation. When RCAN1 was over-expressed in a glioblastoma cell line, it resulted in a decrease in the expression of a number of NFκB target genes including COX-2, IL-8, monocyte chemoattractant protein 1 (MCP-1), ICAM1 and VCAM1 [51]. This study demonstrated that RCAN1 inhibited NFκB signalling through a mechanism that reduced the basal turnover rate of IκBα thereby enhancing its stability [51]. By increasing the level of steady state IκBα, RCAN1 was able to exert anti-inflammatory effects by preventing NFκB activation following stimulation with inflammatory mediators such as TNFα and IL-1β.

Studies have also linked RCAN1 to NFκB signalling via other members of the pathway. For example, RCAN1 is able to negatively regulate the mRNA expression of NFκB inducing kinase (*NIK*) in PC-12 cells [70]. NIK is a member of the MAP kinase family which acts to phosphorylate and activate IκB kinase α (IKKα). Once active, IKKα phosphorylates IκBα, which in turn causes it to dissociate from NFκB, allowing the transcription factor to migrate into the nucleus and activate target genes. If RCAN1 negatively regulates the expression of *NIK*, IκB would remain bound to NFκB and inhibit NFκB signalling [33]. Interestingly, while RCAN1 regulates *NIK* expression, NIK also acts on RCAN1. As mentioned above, NIK phosphorylates the C-terminal region of RCAN1, the end result of which is to reduce RCAN1 proteasomal-dependent degradation and increase the stability of RCAN1 protein [33]. The functional consequences of this increased stability of RCAN1 on NFκB signalling have yet to be determined; however consistent with the study described above [51] it seems likely that elevated levels of RCAN1 would increase the stability of Iκβ which would in turn inhibit the NFκB signalling pathway.

5.5. Angiogenesis

Angiogenesis is a physiological process involving the growth of new blood vessels essential for embryonic development as well as growth and development throughout life. This process has also been associated with disease states including inflammation, tumourigenesis and

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cardiovascular disease [71]. Angiogenesis is orchestrated by a balance between pro-angiogenic factors and angiogenic inhibitors [72]. A critical mediator of angiogenesis is Vascular endothelial growth factor (VEGF) which acts to stimulate angiogenesis and vascular permeability [73-75]. VEGF stimulation of cells causes the rapid activation and translocation of NFAT into the nucleus which in turn results in the up regulation of numerous genes associated with angiogenesis [76]. A number of studies have implicated RCAN1 in angiogenesis. Early studies found that *RCAN1* mRNA increased by 6-fold when endothelial cell lines were treated with VEGF [77, 78] and RCAN1 protein increased in human aortic endothelial cells (HUVECs) similarly treated [49, 79]. RCAN1 gene expression was also up regulated by other mediators of angiogenesis including thrombin [80].

Both major RCAN1 isoforms are involved in angiogenesis and appear to be regulated by different mechanisms. When human endothelial cells were treated with VEGF, there was an induction of *RCAN1-4* mRNA after 30 min, with the highest levels observed after 1 hour. Expression returned to basal levels by 24 hours after treatment [79, 81]. Others reported that up regulation of *RCAN1-4* during angiogenesis was mediated by calcium and calcineurin signalling, because treatment with cyclosporine A (CsA), a calcineurin inhibitor, or intracellular calcium chelators prevented its up regulation [80, 82]. Further evidence to suggest that *RCAN1-4* was regulated by calcineurin signalling came from studies demonstrating that *RCAN1-4* expression following VEGF and thrombin treatment was dependent upon the cooperative binding of transcription factors NFAT and GATA to the *RCAN1-4* promoter [80]. RCAN1-1 expression also appears to be modulated during angiogenesis. While initial studies found that *RCAN1-1* was not induced following VEGF treatment [79, 81], more recent reports have indicated that RCAN1-1 is up regulated in cultured endothelial cells treated with VEGF and during angiogenesis *in vivo* [49, 83]. However, unlike expression of *RCAN1-4* during angiogenesis, *RCAN1-1* expression does not appear to be regulated by the calcineurin signalling pathway as its expression was unaffected by treatment with either CsA or intracellular calcium chelators [80, 82].

A number of reports have suggested that RCAN1-1 and RCAN1-4 may play opposing roles in angiogenesis, where RCAN1-1 appears to be pro-angiogenic and is capable of inducing the formation of new blood vessels, while RCAN1-4 inhibits angiogenesis and vessel formation. For example, siRNA-mediated silencing of *RCAN1-1* in HUVECs inhibited VEGF-induced endothelial cell proliferation and angiogenic responses [49]. Further, when *RCAN1-1* was over-expressed in these cells it induced angiogenesis even in the absence of VEGF. This effect was also observed *in vivo* when human skin melanoma (SK-MEL-2) cells, which over-express VEGF-A, were transfected with *RCAN1-1*, implanted into a matrigel and transplanted into mice. In this situation, exogenous expression of *RCAN1-1* in SK-MEL-2 cells induced angiogenesis and vessel formation [49]. In contrast, RCAN1-4 appears to be anti-angiogenic as over-expression of *RCAN1-4* in SK-MEL-2 cells inhibited angiogenesis and siRNA-mediated silencing of *RCAN1-4* enhanced VEGF-induced proliferation [49]. Another study [80] found that forced up regulation of *RCAN1-4* in primary endothelial cells resulted in a reduction in the expression of many pro-angiogenic genes, including cell cycle inhibitors and growth factors and cytokines involved in the formation of new blood vessels, and moreover, the

formation of tube structures (as a model for blood vessel development) formed from primary human endothelial cells *in vitro* was inhibited. Consistent with this, B16 melanoma cells engineered to over-express *RCAN1-4* and implanted subcutaneously into C57BL6 mice displayed a reduction in tumour growth due to a decrease in blood vessel density [80]. Interestingly, RCAN1-4 is thought to exert its anti-angiogenic effects by providing a negative feedback loop to inactivate calcineurin, preventing nuclear translocation and transcriptional activity of NFAT after VEGF stimulation. In support of this, ablation of *RCAN1-4* expression in endothelial cells increased NFAT activity and was associated with increased transcription of NFAT-regulated genes, such as *E-selectin* and *VCAM1* [78]. Intriguingly, RCAN1-1 was found to activate NFAT activity and enhance is pro-angiogenic functions [49]. Thus, RCAN1-4 inhibits the calcineurin/NFAT pathway while RCAN1-1 activates it.

6. The consequences of RCAN1 over-expression in the DS brain

6.1. Down syndrome and the neural system

DS is the leading genetic cause of intellectual impairment in the general population and is thought to contribute to around 30% of all cases of moderate to severe mental retardation [84]. Mental retardation in DS is characterised by behavioural and cognitive impairments which include low IQ, language deficits and defects in both short and long term memory. Later these deficits are compounded by the early onset of dementia [85].

People with DS exhibit a reduced performance on a number of different tests designed to demonstrate short term or working memory, including visual perception, visual imagery and spatial imagery tasks [86]. Long term memory is also affected by DS with both implicit (defined as improvement in perceptual, cognitive or motor tasks without any conscious reference to previous experience) and explicit (intentional recall or recognition of experiences or information) memory impaired [87]. In addition to the cognitive defects observed throughout life, neuropsychological tests showed that there is a cognitive decline in DS individuals with age and these cognitive changes equate to those observed following the onset of dementia [88]. DS participants with early stage dementia displayed severely diminished long term memory as well as a decreased ability to retrieve stored information compared with the non-demented DS controls [88]. The decline in these forms of cognition, particularly the ability to form new long term memories, is analogues to the cognitive deterioration seen in early to moderate Alzheimer's disease (AD) [89]. Interestingly, the cognitive defects that characterise DS are associated with hippocampal-based learning and memory while prefrontal-mediated executive function and cognition remain relatively unaffected [85].

The cognitive impairments in DS are accompanied by many neuro-morphological changes. Individuals with DS have a significant reduction in brain weight and volume [90], despite brain weight falling within the normal range at birth [91]. DS brains have a shorter anterior-posterior diameter, a reduction in the size of the frontal lobes, a flatter occipital lobe and a smaller brain stem and cerebellum [91]. The anterior and posterior corpus callosum regions and hippocampus are also smaller [92-95]. The hippocampus is a key brain structure involved in learning

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and memory and many of the behavioural and cognitive defects seen in DS are hippocampal-dependent [85]. The difference in hippocampal volume is most likely due to various structural abnormalities, including a decrease in the mean area of the dentate gyrus (DG) and inadequate migration of cells into the pyramidal cell layer [96]. Notably, in adults there is an additional age-related decrease in the volume of the hippocampus, most likely due to some degree of neurodegeneration [95].

Smaller brains in DS individuals probably result from a reduction in the total number of neurones, with certain regions preferentially affected. DS brains exhibit a decrease in neuronal density by adulthood of between 10-50% [91]. The cortex of DS adults exhibits decreases in neuronal number and density in addition to abnormal distribution of neurones [97]. This same pattern of neuronal loss was also observed in the hippocampus and visual cortex. Interestingly, DS foetuses exhibited the same pattern of neuronal development as normal foetuses, with similar neuronal morphology, dendritic spine number and density [98]. However shortly after birth defects were evident and became more pronounced with age [99]. This indicates that something happens after birth which results in alterations in neuronal number and morphology. Using Golgi staining which allows for the visualisation of neurones including their cell bodies, axons, dendrites and spines, the brains of DS infants exhibited shorter basilar dendrites with a significant decrease in the absolute number of spines [100], which was postulated to correlate with a 20-35% decrease in surface area per synaptic contact [91]. Why and how this decline in neuronal development occurs is currently undetermined. These same defects were observed in adults with DS, who exhibited decreased dendritic branching, dendrite length and spine density [101]. Biochemical examination of adult DS brains also revealed a significant reduction in the concentrations of various neurotransmitter markers including, noradrenaline, serotonin or 5-hydroxytraptamine (5-HT) and choline acetyltransferase (ChAT) [102, 103], again signifying neuro-functional deficits in the brain.

On top of the neurodevelopmental problems associated with DS, all individuals with the disorder develop the neuropathological and neurochemical changes associated with AD by the third decade of life [89]. This includes the accumulation of amyloid β (Aβ), formation of hyperphosphorylated Tau-containing neurofibrillary tangles (NFT) and senile plaques. The progression of AD-neuropathology is analogous in both DS and AD, despite occurring decades earlier in DS [104].

6.2. RCAN1 in the brain

RCAN1 has been implicated in development and function of the brain. *Rcan1* is expressed in the developing mouse neural tube from embryonic day (E) E9.5 onwards and at E11.5-E12.5 was detected in the telencephalic vesicles, the caudal hypothalamus, the pretectum and the basal plate of the hindbrain and spinal cord. In later stages of embryonic development, *Rcan1* was highly expressed in the neural proliferative and differentiation zones within the brain with lower expression observed in other regions, including the telecephalon, hypothalamus, pretectum, cortical plate, striatum, amygdala, midbrain, hindbrain and spinal cord. In the post natal brain *Rcan1* gene expression was widely distributed throughout, with the highest levels in the olfactory bulb, the cerebral cortex, hippocampus and dentate gyrus, striatum and septum, amygdala,

hypothalamus and the habenula. Within the hippocampus and dentate gyrus, highest levels of expression were observed in the pyramidal and granular cell layers [105].

Western blot analysis using an antibody designed to detect both RCAN1-1 and RCAN1-4 proteins found that the two isoforms were differentially expressed in the adult mouse brain. RCAN1-1 was abundant throughout the brain, with the highest levels of expression detected in the cortex and hippocampus [20, 54, 106]. RCAN1-4 was generally found at lower levels in the hippocampus, striatum, cortex and prefrontal cortex [54]. Similar results have been observed in the adult human brain where RCAN1-1 was most highly expressed in the cerebral cortex, hippocampus, substantia nigra, thalamus and medulla oblongata [21]. It is worth noting that while one study indicated that both isoforms of RCAN1 were located exclusively within neurones and not in astrocytes or microglial cells [107], another study found a wider distribution pattern [106], with RCAN1-1 and RCAN1-4 detected in multiple cell types including astrocytes and microglia. The highest levels of expression were observed in neurones [106]. Moreover, RCAN1-1 was also detected in primary glial-like cell cultures containing microglial cells and expression of RCAN1-4 was strongly induced following calcium stress [106].

Experimental evidence suggests that RCAN1 has a role in brain function. For example, studies on the RCAN1 orthologue in *Drosophila* known as *nebula*, demonstrated that a loss-of-function mutation of *nebula* displayed a decrease in learning and memory acquisition and performed significantly worse on learning and memory tests after a single trial compared with WT controls. Testing after 1 hour found no difference in the short term memory performance, however tests of long term memory (after 24 hours) found that *nebula*-deficient flies displayed virtually no long term memory [108]. This defect was apparent despite the normal presence of mushroom bodies (the learning and memory centres in *Drosophila*). The decrease in learning and memory observed was attributed to abnormal calcineurin signalling, as *nebula* loss-of-function mutants exhibited a 40% increase in calcineurin activity [108]. Interestingly, over-expression of *nebula* resulted in a similar phenotype. When *Drosophila* over-expressing *nebula* were generated and tested, they displayed virtually no ability to learn. This study also found that transient over-expression of *nebula* was sufficient to cause learning and memory deficits, indicating that a biochemical defect was responsible for learning and memory rather than a pre-existing developmental abnormality [108], a finding that may have implications for DS treatment options.

Similar behavioural abnormalities were observed in RCAN1-KO mice. While the absence of *Rcan1* did not result in any gross anatomical changes within the brain, RCAN1-KO mice exhibited various behavioural and synaptic deficiencies. For example, RCAN1-KO mice were shown to have impaired learning and memory in the Morris Water Maze (MWM), a well-established paradigm of hippocampal-dependent learning and memory. During the acquisition phase of the trial, RCAN1-KO mice displayed a decreased ability to learn the location of the platform compared with WT controls. This indicated that RCAN1-KO mice had a spatial learning impairment. These mice also displayed a poor spatial memory because when the escape platform was removed, RCAN1-KO mice did not demonstrate a specific preference for the target quadrant. On the other hand, a passive avoidance test using electric shock found that long- and short-term contextual fear memory was normal in these mice [54]. Taken

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together results from this study suggested that the absence of *Rcan1* selectively affects some, but not all, types of memory.

These behavioural deficits in RCAN1-KO mice were accompanied by abnormal synaptic transmissions and impaired long term potentiation (LTP). LTP is a form of synaptic plasticity hypothesised to be a biological substrate for some forms of memory [109]. Two forms of LTP can be examined: early-component LTP (E-LTP), a weak and short-lived enhancement of synaptic transmission; and late-component LTP (L-LTP) which is a robust enhancement of synaptic transmission lasting many hours [110, 111]. Paired-pulse facilitation (PPF) is also a component of LTP and is a measure of pre-synaptic short-term plasticity and neurotransmitter release [112]. Absence of RCAN1 did not affect the basal level of synaptic transmission but did result in a reduction in PPF compared with the WT controls, suggesting that pre-synaptic short term plasticity was affected by the lack of *Rcan1*. While there was no difference in the E-LTP, L-LTP was adversely affected by the ablation of *Rcan1*, with RCAN1-KO mice exhibiting a reduction in initial amplitude of L-LTP as well as a reduction in duration of the potentiation [54]. This is significant because the amplitude and duration are the biological correlates of synaptic strength required to reinforce the laying down of memory.

The strongest evidence to suggest a role for RCAN1 in the neurological defects observed in DS comes from a recent study by our group examining *RCAN1* transgenic (RCAN1-TG) mice. Using mice engineered to over-express *RCAN1-1* at a level analogous to that observed in DS, we found up regulation of RCAN1 contributed to some of the neurological defects characteristic of DS. For example, RCAN1 over-expression resulted in multiple defects in the formation, structure and function of the hippocampus [55]. Specifically, there was a significant reduction in the overall size of the hippocampus and analysis of the various structures within the hippocampal formation revealed a decrease in the absolute volume and cellularity of the dentate gyrus [55], mirroring the structural hippocampal defects and marked neuronal loss observed in DS. Our study suggested that the decrease in neuronal cellularity within the hippocampus of RCAN1-TG mice was the result of defective neurogenesis because fewer terminally differentiated neurones within the dentate gyrus formed and progenitor cells isolated and cultured from the sub ventricular zone had diminished ability to differentiate into neurones. This also reflects changes observed in DS [113]. RCAN1 transgenic mice also exhibited neuro-physiological impairments. In particular, over-expression of RCAN1 resulted in a defect in the maintenance phase of LTP which may be explained in part, by the reduction in post-synaptic spine density observed in the brains of these mice. Failure to maintain LTP in hippocampal slices was accompanied by deficits in hippocampal-dependent spatial learning and in short and long term memory. At a molecular level, in response to LTP induction, we observed diminished calcium transients and decreased phosphorylation of CaMKII and ERK1/2, signifying that the processes essential for the maintenance of LTP and formation of memory [55] are defective in mice with an excess of RCAN1.

RCAN1 has also been shown by our group to be involved in neurotransmission. Using chromaffin cells cultured from the adrenal gland as a model for the neuronal system, cells from both RCAN1-TG and RCAN1-KO mice displayed a reduction in neurotransmitter release. Our study demonstrated that the normal function of RCAN1 was to regulate the number of synaptic

vesicles fusing with the plasma membrane and undergoing exocytosis, and the speed at which the vesicle pore opens and closes [53]. Although our study showed that the final outcome was the same whether RCAN1 was in excess or deficit, increased expression of *RCAN1* had the opposite effect to *Rcan1* ablation on vesicle fusion kinetics - ablation slowed fusion pore kinetics while over-expression accelerated fusion pore kinetics.

6.3. RCAN1 in neurodegeneration

Although it has not been proven, there is circumstantial evidence to suggest that RCAN1 plays a role in neurodegenerative conditions (other than DS). For example, Northern blot analysis of human brain samples found that *RCAN1* expression was increased about 2-fold in brains of AD patients [21, 107]. This increased gene expression was confined to the regions of the brain affected by AD, such as the hippocampus and cerebral cortex. This study also found that regions of the brain containing NFT had up to 3 times more *RCAN1* mRNA compared with the same regions of the brain without tangles [21]. Immunohistochemistry on human brain tissue using a RCAN1-specific antibody, found that RCAN1 protein levels increased in abundance with normal ageing in pyramidal neurones with further increases observed in brains affected by moderate to severe AD [65, 107]. In addition to increased protein levels, there was an alteration in the subcellular location of RCAN1 in AD-affected neurones, with a significant increase in the amount of RCAN1 within the nucleus compared with non-diseased tissue [65]. Interestingly, there was an up regulation of RCAN1-1 mRNA and protein in the hippocampus of AD patients, with no changes observed in the abundance of RCAN1-4 [65, 107], suggesting divergent functions of the major isoforms.

While these observations are intriguing, the question remains, what effect does increased RCAN1 expression have on the ageing brain and does it play a role in AD-like neuropathology? While this question remains unanswered, there are a number of possible reasons as to why increased RCAN1 expression might lead to neurodegeneration. One proposed explanation invokes a possible relationship between elevated RCAN1 expression, AD-like neurodegeneration and Tau protein. Tau is involved in the stabilisation of the microtubule networks within neurones and its hyperphosphorylation has been linked to the pathogenesis of AD. Tau can be phosphorylated by a number of different kinases, including GSK-3β and Ca^{2+}/calmodulin-dependent protein kinases (CaMK). Hyperphosphorylation of Tau is detrimental and can lead to AD neuropathology, including formation of NFT [114-116]. During normal cellular processes, there is a proteasome-dependent degradation of Tau protein but when Tau becomes hyperphosphorylated, it is resistant to this degradation and accumulates within the cell [117]. Some studies have found that increased levels of RCAN1 result in a concomitant increase in the phosphorylation of Tau and thus may contribute to its neuronal accumulation [67, 117] and we showed an accumulation of hyperphosphorylated Tau in the brains of aged RCAN1-TG mice [118]. This observed enhancement in Tau phosphorylation may be due to the effect of RCAN1 on GSK-3 activity, since increased RCAN1 expression in PC-12 cells resulted in an increase in the absolute level of GSK-3β, which in turn enhanced its ability to phosphorylate Tau [67]. There have also been suggestions that excess RCAN1 can exacerbate AD-like neuropathology by inhibiting calcineurin. Calcineurin activity is decreased in AD [119] and

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hyperphosphorylated tau protein and cytoskeletal changes in the brain similar to those observed in AD accumulate when the phosphatase activity of calcineurin is reduced [120]. Thus, if RCAN1 is behaving as a calcineurin inhibitor it is possible that increased levels of RCAN1, as occurs in DS and AD, promote the development of AD [21] [121].

RCAN1, via its role as an inhibitor of calcineurin, has also been implicated in the pathogenesis of Huntington's disease (HD). In a mouse model of HD, phosphorylation of huntingtin at serine residue 421 was protective and treatment of HD neuronal cells with calcineurin inhibitors prevented their death by maintaining their phosphorylation status at Ser^{421} [122]. RCAN1-1L protein was significantly down regulated in human HD post mortem brains and exogenous expression of RCAN1-1L in a cell culture model of HD protected the cells against toxicity caused by mutant huntingtin [123]. This protection was attributed to the ability of excess RCAN1 to inhibit calcineurin phosphatase activity, indicating that in this circumstance RCAN1 over-expression is advantageous.

Another connection between RCAN1 and neurodegeneration may be through the formation of aggregates. When proteins accumulate within a cell a mitrotubule-based apparatus known as an aggresome acts to sequester proteins within the cytoplasm. The formation of aggresomes within cells is most likely a defence mechanism against the presence of misfolded or abnormal proteins. However if these misfolded proteins are not cleared appropriately it can lead to abnormal protein accumulation and eventual neurotoxicity [124]. The formation of aggresomes is believed to contribute to many neurodegenerative disorders including AD, Huntington's disease and cerebral ataxia [125]. When RCAN1 was over-expressed in various neuronal cell lines and in primary neurones, formation of aggregates occurred [124] and the aggregates were associated with microtubules, indicating that they had formed inclusion bodies within the cells. When RCAN1 was aggregated within neurones, neuronal abnormalities characterised by a decreased number and density of synapses were observed, which in turn altered synaptic function [124]. This constitutes another example of the damaging effects of excess RCAN1.

Finally, two polymorphisms located in the *RCAN1-4* promoter region have been associated with AD in the Chinese Han population [126]. One of these, rs71324311, in the heterozygous-deletion genotype confers protection while the other, rs10550296, also in the heterozygous-deletion configuration, is a risk factor. The functional consequences of these sequence variants are yet to be determined.

7. The consequences of RCAN1 over-expression in the DS immune system

7.1. The Down syndrome immune system

DS is associated with a multitude of immune system defects. People with DS are more susceptible to infections, particularly respiratory tract infections with pneumonia one of the major causes of early death [127]. The incidence of viral hepatitis and haematopoietic malignancies is also increased in people with DS as is their tendency to develop certain types of

autoimmune disorders such as autoimmune thyroid disease (AITD) (Hashimoto type), coeliac disease and diabetes [127] [128]. Thus, DS appears to include a combination of immunodeficiency and immune dysfunction. Although the precise cause of this immune dysfunction is unclear, the DS immune system is characterised by a number of abnormalities thought to originate from defective innate and adaptive immunity.

7.2. Impairments in innate immunity

Innate immunity is the body's first line of defence against invasion. This arm of the immune system either prevents the entry of pathogens into the body, or upon entry, eliminates them before they can cause any damage or disease. If a pathogen is able to gain entry into the body, innate immunity includes various non-specific mechanisms which can eliminate and destroy foreign invaders. These mechanisms include phagocytosis and inflammation. DS is associated with defects in the innate immune system. For example, natural killer (NK) cells, components of the innate immune system involved in the recognition and elimination of bacteria, viruses and tumour cells, are defective in DS individuals [129]. Also, neutrophils from DS people exhibited a decreased ability to phagocytose [130] and the ability of DS-derived neutrophils and monocytes to migrate towards a site of injury or infection in response to chemokine release was reduced [131].

7.3. Impairments in adaptive immunity

T cell development and maturation occurs within the thymus. Bone marrow (BM) derived precursor cells migrate into the thymus where they receive developmental cues from the thymic microenvironment. Here they progress through a number of different stages of development broadly defined by the expression of CD4 and CD8 on the cell surface. Once cells become fully mature, expressing only CD4 or CD8 on the surface, they are able to migrate to the periphery and populate the immune system. The DS immune system is characterised by a number of abnormalities thought to originate from defective T cell development in the thymus. Typically, the DS thymus is small and morphologically abnormal. It exhibits cortical atrophy, loss of cortico-medullary demarcation and lymphopenia due to a defect in the development of thymocytes [114]. The number of cells expressing high levels of the TCR α-β-CD3 complex is reduced [132] as are the numbers of helper ($CD4^+$) T (Th) cells resulting in the inversion of the normal $CD4^+/CD8^+$ ratio in favour of the $CD8^+$ population. Th cells can be further subcategorised into either Th1 or Th2 cells where Th1 cells participate in the elimination of intra-vesicular pathogens, including bacteria and parasites via the activation of macrophages, while Th2 cells clear extracellular pathogens and toxins by assisting antibody production in B cells. There is an imbalance in the T helper responses of DS individuals, although there is some disagreement as to whether it is an alteration in the Th1 or Th2 phenotype. Some studies have suggested that Th2 responses are augmented in DS based on the observation that there is an increased number of circulating $CD3^+$/CD30 Th2 lymphocytes [133]. Others report an increase in the Th1 population in DS and this has been attributed to increased IFNγ production [134] because IFNγ polarises Th0 cells towards the Th1 phenotype. While there is no doubt that a defect in T cell development and maturation within the DS thymus exists,

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altered apoptosis of lymphocytes may also contribute to the decrease in overall numbers of T cells in the periphery, as well as to the alterations observed in the abundance of the various T cell subsets. For example, DS $CD3^+$ T cells and $CD19^+$ B cells expressed significantly higher levels of early apoptotic markers compared with control cells [135].

T lymphocytes isolated from DS people are also functionally compromised. Under conditions designed to simulate an infection using anti-CD3 antibodies or the non-specific mitogen, phytohemagglutinin to activate T cells, DS lymphocytes were diminished in their proliferative capacity [136, 137]. Not only did the DS-derived T cells have a proliferative defect, they showed increased expression of apoptotic markers including APO-I/Fas (CD95) antigen, a T cell death marker, and increased apoptosis was demonstrated in cultured T cells using Annexin V [138]. $CD8^+$ or cytotoxic T lymphocytes (CTLs) isolated from DS individuals were also compromised in their ability to kill target cells [139], indicating a functional defect in this cell type also. DS-derived T cells also produce abnormal levels of cytokines, the small proteins produced by immune cells that are involved in signalling and controlling immune responses. IL-2 is central to the proliferation and differentiation of T cells and is produced by T lymphocytes once activated. Inhibition or reduction in IL-2 results in suppression of the immune system. One study on adults with DS found that the levels of IL-2 secreted from cultured stimulated T cells were significantly reduced compared with T cells cultured from normal individuals [140]. Other studies have suggested that IL-2 is produced at comparable levels in both DS and normal individuals, but in DS the response to IL-2 may be defective [141]. Levels of IFN-γ and TNF α are also altered in DS and although the number of DS studies is small, the consensus is that IFN- γ and TNF α levels are increased [142] [134].

In addition to T cell lymphopenia, DS individuals have marked B lymphopenia [143-145]. As well as a reduction in the number and proportions of B lymphocytes, there is a skewing of the B cell subpopulations, suggesting that maturation of B cells is defective in DS [146] akin to the situation with T cells, although the exact nature of this defect has not been explored. Immunoglobulin levels in DS are also abnormal, with DS B lymphocytes producing lower levels of IgM, IgG_2 and IgG_4 and higher levels of IgG_1 [146, 147]. IgG_3 and IgA levels were unchanged. Also suggesting a B cell functional deficit is the finding that antibody responses to a variety of antigens are low in DS, including the responses to pneumococcal and bacteriophage ØX174 antigens and to vaccine antigens such as tetanus, influenza A and polio [148-150].

7.4. RCAN1 in innate immunity

There is evidence to indicate that RCAN1 has a role in innate immunity and inflammation. For example, when human mononuclear cells were activated with *Candida albicans*, a pathogen capable of eliciting an innate immune response, RCAN1 gene expression was rapidly induced [151]. RCAN1 expression was also induced in response to various pro-inflammatory cytokines involved in the innate immune system such as TNFα [78]. Other studies have found that RCAN1 regulates inflammatory mediators and cytokines that have previously been identified as components of the innate immune system. For example, forced over-expression of RCAN1 in endothelial cells using adenoviral vectors resulted in a decrease in the expression of inflammatory markers such as E-selectin, VCAM1, TNF and COX-2 mRNA [78]. This sug-

gested that increased expression of RCAN1 may dampen inflammation and inhibit induction of the innate immune system. Conversely, knockdown of RCAN1 using siRNA resulted in an increase in the expression of inflammatory mediators [78].

Importantly, RCAN1 also mediates inflammatory responses *in vivo*. When mice were administered with lipopolysaccharide (LPS), a component of gram negative bacteria cell wall used experimentally to activate innate immune responses, *Rcan1* gene expression was induced [152]. Interestingly, RCAN1-KO mice had lower survival following LPS-induced endotoxaemia compared with their WT littermates [152]. Knockout mice had an accentuated response to LPS treatment, including lower heart rate, blood pressure and body temperature. An increase in the concentration of circulating IL-6 protein, a pro-inflammatory cytokine believed to be detrimental during infection was also found, along with a significant increase in the mRNA expression of inflammatory mediators such as *E-selectin*, *ICAM1* and *VCAM1* in organs including the heart and lung. There was a concomitant increase in the number of infiltrating leukocytes within these organs [152]. On the other hand, over-expression of *RCAN1-4* achieved by the intravenous injection of mice with a *RCAN1-4*-containing adenovirus, conferred a survival advantage upon LPS administration. A decrease in the levels of circulating IL-6 and an attenuation of the physiological responses to systemic LPS treatment were evident [152]. Induction of inflammatory mediators was also reduced and there was a marked reduction in leukocyte infiltrate in the heart, liver and lungs [152]. Another study found that following infection with the bacteria *Fransicella tularensis*, induction of pro-inflammatory cytokines including MCP1, IL6, IFNγ, and TNFα was significantly higher in *Rcan1*-deficient spleen and lung [153]. All this suggests that over-expression of RCAN1 is protective.

Other studies on the role of RCAN1 in innate immunity have focussed on identifying the mechanisms by which RCAN1 regulates inflammation. One plausible means is by modulation of the NFκB signal transduction pathway. As described earlier, RCAN1 is able to inhibit NFκB signalling by increasing the stability of IκB protein [51]. Given that NFκB is a transcription factor that controls the expression of pro-inflammatory genes and the subsequent activation of innate immune cells, negative regulation of this pathway by RCAN1 would result in inhibition of inflammation. Such a proposition is consistent with published *in vitro* and *in vivo* data. However, another study investigating the potential involvement of RCAN1 in the Toll-like receptor (TLR) pathway arrived at the opposite conclusion [154]. The TLR pathway is activated as a first line defence mechanism during microbial infection and culminates in the induction of interleukins and other pro-inflammatory mediators [155]. When RCAN1-4 (DSCR1-1S) was exogenously expressed in HEK293 cells, the end result was activation of NFκB-mediated inflammatory responses [154], not suppression. Here, RCAN1 was found to regulate the TLR pathway through a direct interaction with the adaptor protein known as Toll-interacting protein (Tollip). The normal cellular role of Tollip is to suppress TLR signalling by sequestering IL-1 receptor associated kinase 1 (IRAK-1). Exogenously added RCAN1 bound Tollip, causing the release of IRAK-1 from the complex thereby removing the block on IRAK-1 activity [154]. The end result was an enhancement of the inflammatory response and thus represents yet another example of the sometimes contradictory actions of RCAN1.

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7.5. RCAN1 in adaptive immunity

The first evidence to suggest that RCAN1 functions in adaptive immunity came from experiments investigating T cell responses in human Jurkat cells, an immortalised T lymphocyte cell line. When these cells were stimulated with the T cell mitogens, CD3 and CD28, expression of *RCAN1-4* mRNA was induced [26]. This result was confirmed by stimulating primary T cells cultured from humans [156]. A more definitive role for RCAN1 in the adaptive immune system came from examining RCAN1-KO mice [44]. While these mice displayed normal T cell development and maturation with comparable numbers of mature thymocytes and equivalent numbers of $CD4^+$, $CD8^+$, $CD3^+$ T cells in the periphery, these cells exhibited functional deficits. When the T cells were isolated from the spleen and cultured *ex vivo*, the RCAN1-KO cells were functionally defective. Specifically, these T cells exhibited a 50% reduction in proliferation in response to mitogenic stimulation as well as a decrease in the production of IFNγ. This loss of IFNγ indicated that the Th1 population was especially affected by the lack of *Rcan1* expression. Indeed, these mice exhibited defective Th1 responses due to the premature death of this population of cells as a result of an up regulation of FasL and a loss of viability. Antibody class switching was also altered in RCAN1-KO mice, with a decrease in IgG_2 production. Notably, the T cell defect in RCAN1-KO mice could be rescued by treatment with the calcineurin inhibitor, CsA, suggesting that the defect was calcineurin/NFAT-dependent and presumably due to hyperactivation of the calcineurin signal transduction pathway [44]. However, despite restoration of T cell function in RCAN1-KO mice following CsA treatment, genetic loss of calcineurin Aβ superimposed on the *Rcan1* deficiency by crossing RCAN1-KO mice with CnAβ knockout mice, could not rescue the T cell defects [64]. In fact, loss of calcineurin Aβ in addition to the loss of *Rcan1* resulted in an increase in the severity of the T cell defect. This observation suggests that in these mice RCAN1 is acting to facilitate calcineurin activity rather than inhibit it as the use of CsA treatment had suggested. Our group also has evidence of RCAN1's involvement in adaptive immunity; our RCAN1-TG mice have T and B cell defects (unpublished data and manuscript in preparation).

In addition to its function in T cells, RCAN1 is involved in the normal function of mast cells. Mast cells are specialised immune cells that contain granules rich in histamine and heparin and are known to play a role in wound healing, defence against pathogens and the pathology of IgE-dependent allergic disease and anaphylaxis [157]. Mast cells are activated through the high affinity IgE receptor (FcεRI) on their cell surface and this activation is controlled by a number of activating and inhibitory molecules. The down regulation of mast cell activity by inhibitory signals is essential in preventing allergic disease and anaphylaxis [157]. RCAN1 is believed to be one of these inhibitory signals. Evidence to suggest this comes from experiments conducted on RCAN1-KO mice, which displayed an exaggerated mast cell response. While RCAN1-KO mice displayed normal mast cell maturation, many of the signalling pathways following mast cell activation were perturbed. For example, mast cells isolated from RCAN1-KO mice and stimulated with FcεRI had an increase in the activation of both the NFAT and NFκB signalling pathways. As expected, there was also an increase in the expression of many pro-inflammatory genes regulated by these two pathways including *IL-6*, *IL-13* and *TNFα* [158]. Further, when mice lacking *Rcan1* were sensitised with an intravenous injection of anti-IgE antibody and then later treated with an agent designed to elicit an anaphylactic reaction, *Rcan1* deficiency led to

enhanced mast cell activation, degranulation and passive cutaneous anaphylaxis [158]. These results indicate that RCAN1 may be an inhibitor molecule that negatively controls mast cell function.

Eosinophils, another immune cell type, are predominant effector cells in allergic asthma and their presence in the lungs of asthma sufferers is regarded as a defining feature of this inflammatory disease. Absence of *Rcan1* was shown to prevent experimentally-induced allergic asthma in a mouse model due to an almost complete absence of eosinophils infiltrating the lungs [159]. Although the exact mechanism for this protection is not fully understood, it seems that a lack of *Rcan1* blocks the development and migration of eosinophil progenitors from the bone marrow and selectively lowers their production of the inflammatory mediator IL-4. This study implies that over-expression of RCAN1 would exacerbate the allergic response and in this regard it is interesting to note that a recent study reported an increased incidence of allergic asthma in people with DS [160]. Therefore, it would be very informative to test allergic asthma responses in RCAN1-TG mice.

8. The consequences of RCAN1 over–expression on the incidence of solid tumours in DS

8.1. Down syndrome and cancer

Individuals with DS are more likely to develop certain malignancies, especially of the immune system. There is a well-established link between leukaemia and DS, with an increased incidence in DS compared with the general population. Large population based studies conducted in different countries around the world have consistently found that the rates of leukaemia were between 10- to 19-fold higher in people with DS in comparison with the average population and there was an increased incidence of both lymphoid and myeloid leukaemias [140, 161-163]. While the incidence of both acute myeloid leukaemia (AML) and acute lymphatic leukaemia (ALL) was significantly higher in DS subjects than expected in the general population, there were significantly more cases of AML compared to ALL in DS [163]. This increased risk is most evident at a younger age, however remained throughout life. There is also a significant increase in the incidence of neoplastic disorders such as megakaryoblastic leukaemia, where the incidence is increased about 500-fold in DS [164, 165]. In males, there is also a link between DS and testicular cancer, possibly due to higher levels of follicular stimulating hormone, hypogonadism or cryptorchidism [166, 167]. Notably, those with DS are less likely to develop other solid tumours such as neuroblastomas and breast and lung cancers [162, 163, 168]. Indeed, DS individuals had a 50% reduction in the incidence of solid tumours compared to the number of cases expected in the general population and this was observed over all age groups examined [162]. Thus it seems likely that a number of tumour suppressor genes reside on *Hsa21*.

8.2. RCAN1 and tumourigenesis

While the identities of the *Hsa21* genes responsible for the reduction in solid tumour formation in DS remain unknown, there is evidence to suggest that up regulation of RCAN1 may afford

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some protection. Firstly, a number of cancers display abnormal expression of RCAN1 and this expression varies depending on the stage of the cancer. For example, studies have shown that RCAN1 is up regulated in most primary papillary thyroid tumours but this expression is lost in the metastatic tissue of thyroid tumours [169]. This is interesting given that RCAN1 has been identified as a target gene for metastatin, a protein that functions to suppress metastatic tumour growth. It is possible that loss of metastatin in tumour cells leads to a loss of RCAN1 expression which may in turn contribute to tumour metastasis [169]. RCAN1 has also been linked to other cancers including colorectal cancer. Peroxisome proliferator-activated receptor γ (PPARγ) is a member the nuclear hormone receptor family of transcription factors and has been identified as a tumour suppressor gene in colon cancer. This gene is important in a number of cellular processes including inflammation, proliferation, apoptosis as well as adipocyte and intestinal epithelial cell differentiation and has been shown to suppress experimental colon carcinogenesis in mice (reviewed in [170]). Loss of *RCAN1-4* in *MOSER colon carcinoma cells* resulted in an inhibition of PPARγ-mediated tumour suppression and increased tissue invasion [171]. While not conclusive, these results indicate that RCAN1 may be required for PPARγ suppression of colorectal cancers [171]. Again this is consistent with the idea that RCAN1 can act as a tumour suppressor.

The strongest genetic evidence to suggest a role for *RCAN1* in tumourigenesis comes from experiments conducted on RCAN1-KO and RCAN1-TG mice. When RCAN1-KO mice were injected subcutaneously with renal carcinoma or colon carcinoma tumour cells, there was a significant suppression of tumour growth [172]. Tumour growth was suppressed due to an inability to form and maintain tumour vasculature within the solid tumours. Further investigation showed that RCAN1-KO mice had hyperactive VEGF-calcineurin-NFAT signalling, which resulted in a suppression of endothelial cell proliferation and an increase in apoptosis [172]. Tumour growth in the RCAN1-KO mice could be restored following treatment with CsA, suggesting that suppression of tumour cell growth in RCAN1-KO mice was dependent on hyperactive calcineurin signalling. Perhaps counterintuitively, but similar to the situation with the RCAN1-KO, mice over-expressing *RCAN1-4* were also resistant to tumour growth when injected subcutaneously with Lewis lung or B16F10 tumour cells [173]. Tumours isolated from these mice also displayed a decrease in the density of microvessels and the vessels lacked a functional lumen. Moreover, it appeared that *RCAN1-4* mediated tumour growth through the calcineurin pathway as *RCAN1-4* transgenic tumour cells had a decrease in both calcineurin and NFAT activity [173]. The exact mechanisms by which RCAN1 suppresses solid tumour growth remain unknown, but both studies strongly suggest that regulation of angiogenesis by RCAN1 underpins the inhibition of tumour growth by reducing the formation of blood vessels throughout the tumour. It is interesting to note that RCAN1-KO and RCAN1-TG mice displayed a similar phenotype, with both exhibiting a decrease in tumour formation due to an inhibition of angiogenesis preventing the formation of microvessels required to support tumour growth. Perhaps more intriguing is that opposite effects on the calcineurin pathway produced the same end result. Also intriguing is that microvessel formation was also decreased in teratomas generated from human DS-derived pluripotent stem cells transplanted into WT mice, indicating that decreased angiogenesis may be responsible for tumour suppression in DS [173].

Finally, the significance of RCAN1 in tumour suppression in DS was elegantly demonstrated using yet another DS genetic model. TS65Dn mice that harbour a third copy of many *Hsa21*-orthologous genes, including *Rcan1*, were bred with RCAN1-KO mice, thereby returning the gene dosage of *Rcan1* to normal. When tumour cells were injected into these mice, there was a significant increase in the formation of microvessels within solid tumours compared with their TS65Dn littermates expressing 3 copies of *Rcan1* [173]. This is more evidence to support the idea that elevated levels of *RCAN1* are responsible, at least in part, for the decrease in the incidence of solid tumour formation in DS.

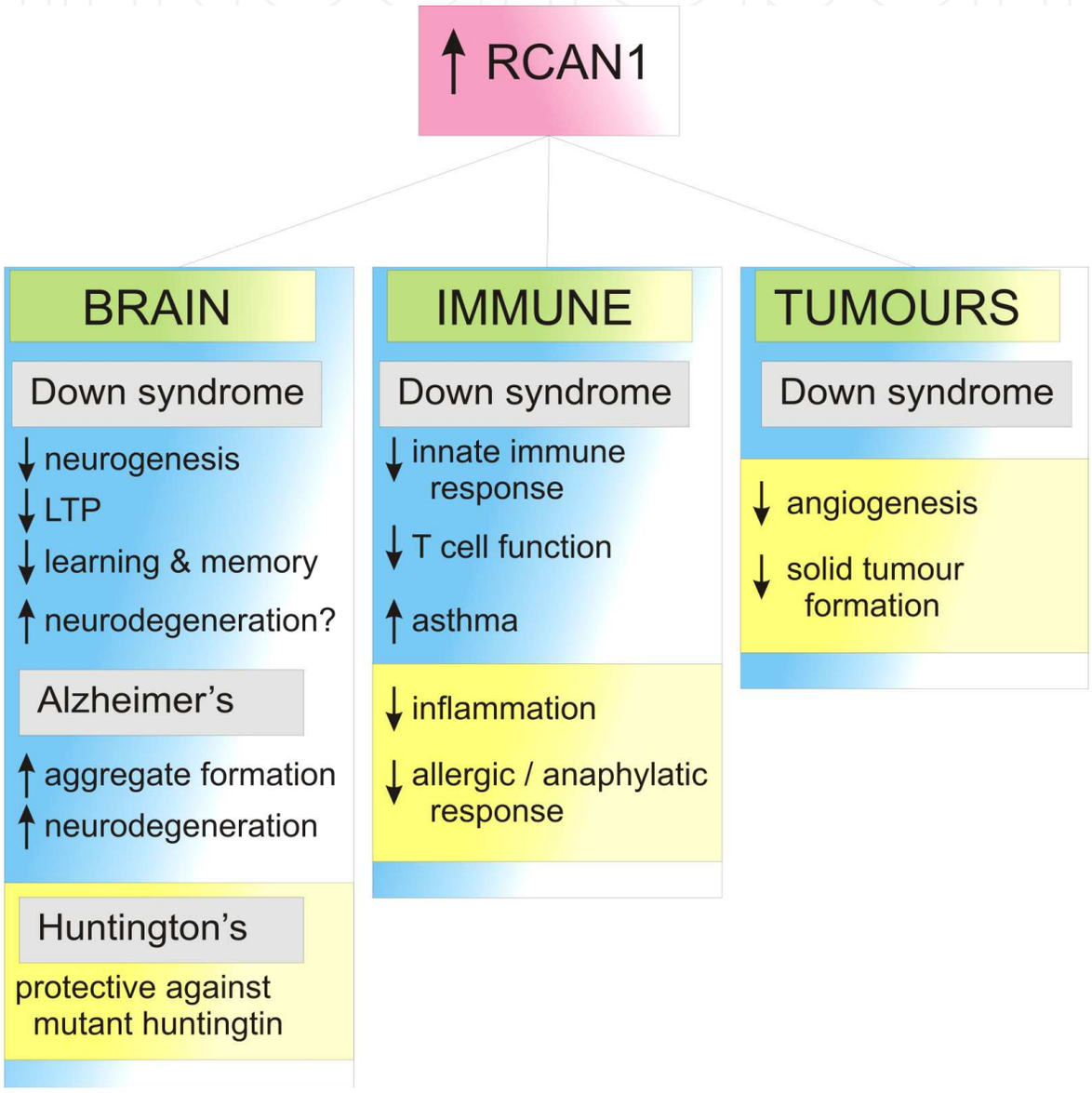

Figure 2. Summary of the positive and negative effects of excess RCAN1. Effects on the brain, immune system and solid tumour formation in Down syndrome are shown. The putative contributions of an over abundance of RCAN1 have either been demonstrated in mouse models or in cell lines or implied from *Rcan1*-KO studies where, in the absence of data to the contrary, the assumption is that over-expression will produce the opposite effect to the deficiency. Detrimental effects are shown in blue and protective effects in yellow.

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9. Conclusion

In this review we have attempted to summarise what is currently known about the function of the *RCAN1* gene and its pleiotropic actions in three areas of relevance to DS (see Figure 2). No matter which system you look at, the reports on RCAN1 function are often contradictory – we still have much to learn. Researchers with a passionate interest in DS and its molecular genetic aetiology have suggested that specific down regulation of a few of the genes produced in excess in DS tissues may provide an avenue for therapies. We and others have suggested that inhibition of RCAN1 signalling may have pharmacological potential for reducing neuronal loss and treating cognitive decline in DS and AD, but we still have much to learn about the molecular function and physiological role of RCAN1 and how we can manipulate its activity to ameliorate/treat pathology.

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