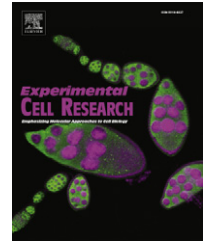


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Review Article

The reticulons: Guardians of the structure and function of the endoplasmic reticulum

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ABSTRACT

The endoplasmic reticulum (ER) consists of the nuclear envelope and a peripheral network of tubules and membrane sheets. The tubules are shaped by a specific class of curvature stabilizing proteins, the reticulons and DP1; however it is still unclear how the sheets are assembled. The ER is the cellular compartment responsible for secretory and membrane protein synthesis. The reducing conditions of ER lead to the intra/inter-chain formation of new disulphide bonds into polypeptides during protein folding assessed by enzymatic or spontaneous reactions. Moreover, ER represents the main intracellular calcium storage site and it plays an important role in calcium signaling that impacts many cellular processes. Accordingly, the maintenance of ER function represents an essential condition for the cell, and ER morphology constitutes an important prerogative of it. Furthermore, it is well known that ER undergoes prominent shape transitions during events such as cell division and differentiation. Thus, maintaining the correct ER structure is an essential feature for cellular physiology. Now, it is known that proper ER-associated proteins play a fundamental role in ER tubules formation. Among these ER-shaping proteins are the reticulons (RTN), which are acquiring a relevant position. In fact, beyond the structural role of reticulons, in very recent years new and deeper functional implications of these proteins are emerging in relation to their involvement in several cellular processes.

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Introduction

Reticulons (RTNs) are a novel class of membrane-bound proteins present in all eukaryotic organisms. RTNs not only associate with ER but also with other cellular constituents including plasma membrane and the Golgi apparatus [1].

In mammals, four independent reticulon genes have been identified and named as *rtn1*, 2, 3, and *rtn4/nogo*. Each of them encodes for different protein isoforms by using alternative splicing sites or different promoter regions [2]. All members of RTN family are characterized by a C-terminal reticulon homology domain (RHD) of 150–200 amino-acid residues, a common structural feature which is evolutionarily preserved from plants to yeasts to humans (Fig. 1). The RHD consists of two hydrophobic regions, that assess the membrane association of the protein, separated by a 66 amino-acid hydrophilic loop and followed by a short C-terminal tail [3]. The conservation of RHD confers common functions among RTNs, such as subcellular localization and/or protein interactions. In fact, unlike ER-resident proteins, reticulons do not display the canonical ER-localization signal in the amino-terminal part but the retention in the ER membrane compartment could be due to the structural conformation of RHD. In fact, disruption of the second transmembrane segment has been observed to affect the ER localization of RTN4 [4], and mutations in the first transmembrane domain of RTN3 are able to compromise its membrane insertion [5]. Moreover, RHD hydrophobic regions are unusually long, around 28–36 amino-acids, in comparison to the alpha-helix domain of typical transmembrane proteins that are

only about 20 amino-acids in length. Therefore, the topology of these hydrophobic regions within membranes diverges from the usual integral membrane proteins [6], and still remains partially elusive. In one of the most qualified model the reticulon transmembrane domains do not fully cross the membrane but each of them forms a hairpin-like structure into the outer leaflet of the lipid bilayer with both N- and C-terminal ends facing in the cytosolic side (“W” topology). These hairpin-like topologies are typical of proteins involved in vesicle budding/membrane-shaping such as caveolin-1 and reggie-1 [7]. Because RTNs are present in cell membranes characterized by high positive curvature, such as ER, it was hypothesized that reticulon hairpin-like structures are responsible for this membrane bending. Consistent with this hypothesis, in *Arabidopsis thaliana* it was demonstrated that the lengths of the transmembrane segments in RTNLB13 are likely to be responsible for its ER membrane curvature [8]. Subsequently, together with RTNLB13, four other members of the reticulon family, RTNLB1-4, have shown a similar effect on the ER structure in *A. thaliana* [9].

It seems that RTNs not only individually bend the membrane but can also form multimeric, arc-like structures which may ultimately shape and determine the diameter of ER tubules [10].

Moreover, it has been demonstrated that the yeast RTN Rtn1p is sufficient to induce tubule formation in vitro when mixed with lipids [11]. Furthermore, similar results from Hu et al. have previously been obtained for RTN4A where its ability to form ER tubule structures was demonstrated in association with DP1, an integral membrane protein orthologues of yeast Rtn1p-binding protein Yop1 [12]. On the other hand, a growing line of evidence supports

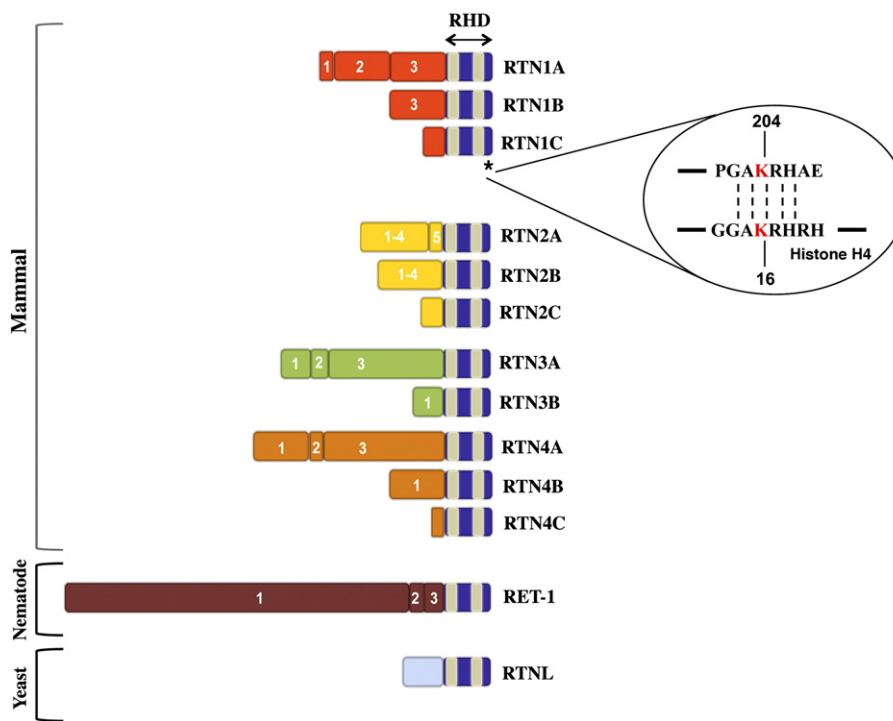


Fig. 1 – Schematic representation of mammal, nematode and yeast RTNs. Numbers indicate the different exons for each reticulon at the N-terminus. The reticulon homology domain is indicated by a gray box and the blue boxes represent the hydrophobic regions. Asterisk refers to RTN1C C-terminal domain (a.a. 201–208) sharing the GAKRHR motif with Histone H4. The acetylated lysine is marked in red.

other different reticulon topologies from the hairpin-like model that might mirror a non-structural role for these isoforms. Thus, it is plausible to think that reticulons can have different topologies when associated to different cellular membrane sites and/or protein partners reflecting the multiple functions exerted by these proteins.

In contrast to the closely conserved C-terminus that may confer common functions among reticulons, the N-terminal regions show little or no sequence homology (Fig. 1) [13]. These variable domains are likely to interact with distinct proteins and to confer specific biological functions to the various reticulon isoforms. In particular, the specific RTN4/Nogo function has been well characterized in mammalian central nervous system (CNS), where it exerts its inhibitory effect in neurite outgrowth [14]. Albeit a great pool of this reticulon protein localizes in the endomembrane system of the cell (ER and Golgi complex), as well as the other RTN family members, a little fraction of Nogo is also expressed in oligodendrocyte plasma membrane where it may function as a CNS-myelin specific inhibitor of axonal regeneration [15]. Unlike the myelin of the peripheral nervous system, the myelin from the CNS prevents neuronal regeneration after injury, and Nogo represents one of the myelin-associated inhibitory factors implicated in this phenomenon. Indeed, *in vivo* neutralizing antibody against Nogo results in axonal growth of corticospinal tract and enhanced functional recovery following spinal cord injury, as well as in sensory motor cortex lesioned rats [16,17].

In particular, two extracellular exposed regions of Nogo, such as a stretch at N-terminus and 66 amino-acid loop of RHD (Nogo-66), are responsible for neurite growth-inhibiting effect [14]. It is now known that Nogo-66 binds NgR, a corresponding receptor present on neuron surface. The activation of NgR and its co-receptors induces the inhibitory signal in neurons via RhoA-ROCK signaling pathway that leads to cytoskeleton rigidification and growth cone collapse [3,18].

Numerous studies have largely demonstrated that blocking Nogo/NgR1 signaling could be a way to promote regeneration [18], and thus Nogo could be a therapeutic target for CNS injuries and neurodegenerative disorders.

Further, more recently four WW domains that can bind to E3 ubiquitin-ligases WWP1 through its PPxY motifs have been identified in the N-terminus of NogoA [19]. On the basis of these

speculations, ubiquitinylation likely represents an important mechanism by which NogoA expression and activity may be regulated.

Reticulons in membrane trafficking

Although reticulons have mainly an ER localization, these proteins are also localized with the Golgi and plasma membrane. This pleiotropic localization, in addition to the reticulon membrane-bending property, suggests an important involvement of RTNs in membrane vesicle trafficking.

Consistent with the implication of reticulons in cellular trafficking, RTN3-overexpression in HeLa cells prevents the retrograde transport of proteins from Golgi to ER [20]. Furthermore, by yeast two-hybrid screening RTN1-A/B have been identified to bind AP50, a component of the AP-2 adaptor complex that mediates endocytosis [21]. Conversely, RTN1C may be involved in exocytosis. In fact, ectopic expression of RTN1C in PC12 cells induces an increase in the secretion rate of the growth hormone [22]. Furthermore, RTN1C has been observed to co-immunoprecipitate with components of the SNARE complexes such as syntaxin-1, -7, -13, and VAMP2 [22] (Fig. 2). Moreover, the *Caenorhabditis elegans* reticulon interacts with the protein RME-1, a regulator of endocytic recycling, thus suggesting an early evolution of the reticulon function in vesicle trafficking of eukaryotic cells (Fig. 2).

Interestingly, it is well known that abnormalities in exocytosis and endocytosis may be one of the mechanisms involved in neurodegenerative diseases. In keeping with this hypothesis, altered expression levels of reticulons have been observed in several neurological disorders (Fig. 2) [23,24]. For instance, RTN3 expression is increased in cerebral tissue obtained from Alzheimer disease patients [25]. Moreover, RTN3 and RTN4B/C have been identified to interact and negatively modulate beta-secretase BACE1, a protease directly involved in amyloidogenic pathway in Alzheimer pathology [26]. However, the molecular basis of how RTN3 and other reticulons may function as a negative regulator of amyloid- β production is unclear. Interestingly, Shi et al. have observed a reduced level of amyloid- β deposition in RTN3-overexpressing mice due to a major retention of BACE1 in the ER compartment where this enzyme is less active. Considering the involvement of reticulons in vesicle trafficking, the authors hypothesize that the

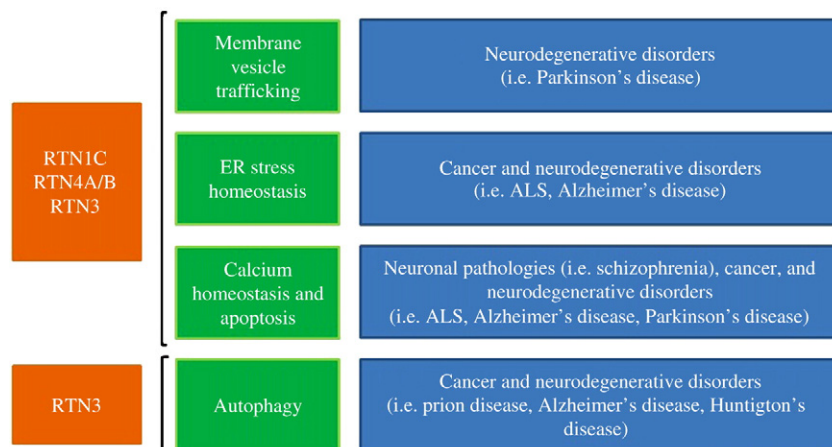


Fig. 2 – Correlation between some reticulon cellular functions and their potential pathological implication.

association of RTN3 with BACE1 alters its trafficking itinerary to the cell surface [27].

Recently, it has been proposed that during cell mitosis, the nuclear envelope is regenerated from the ER and it is not assembled from the fusion of separate vesicles [28]. Notably, Anderson and Hetzer in their recent work have demonstrated that the displacement of ER shaping proteins, such as reticulons, is rate-limiting for nuclear assembly, suggesting a critical transition step from tubular to sheet nuclear membrane. In fact, overexpression of RTN3, RTN4 and DP1 in U2OS cells delays nuclear envelope formation, whereas their knockdown by siRNAs accelerates this process [29]. Moreover, yeast RTN1 and Yop1 are required for nuclear pore complex (NPC) distribution, structural stability and biogenesis. In fact, *rtn1Δ yop1Δ* double mutant results in clusters of aberrant NPC-like structures. Then, in a cell-free assay based on *Xenopus laevis* egg extracts, a specific antibody against N-terminal domain of RTN4A is able to inhibit de novo nuclear pore assembly without affecting the nuclear envelope expansion. This effect could suggest a putative distinct function of the reticulon protein in the nuclear envelope flattening during mitotic *telophase* [30] independently by its role in the nuclear pore formation. In keeping with RTNs involvement in NPC biogenesis and stability, Chadrin et al. found a little pool of RTN1 copurifying with Pom33, a novel transmembrane nucleoporin protein identified in yeast. This data might suggest a dynamic interaction between RTN1 and Pom33, or could be explained by hypothesizing that only the NPC-associated fraction of reticulon takes part in this interaction [31]. In the future, it will be interesting to investigate whether the RTN proteins interact with other NPC components, and to evaluate the possibility that this functional connection is conserved in mammals considering the complexity in the redundant and specific functions of the wide set of RTN isoforms present in these organisms.

Reticulons in cell death

Among the proposed specific functions of RTNs, there are several evidences indicating their implications in the cell death pathways, in particular in the regulation of ER-stress-induced apoptosis [3] (Fig. 2). Considering the role played by the reticulon proteins in ER morphogenesis and homeostasis, it was obvious to think about the important implications of these proteins under stressful conditions of this cellular organelle. It is well known that cell death can be triggered from various organelles, for instance via endoplasmic reticulum. Moreover, ER maintains intimate liaison with other organelles, such as nucleus, Golgi apparatus and mitochondria, and thus represents a central player in cell physiology [32]. In particular, several important regulators of apoptosis, including Bcl2 family members are localized in ER, and their pro/anti apoptotic functions are mediated by an altered signaling from ER to mitochondria, which induces cell death by modulating the release of calcium from the ER store [33].

Interestingly, both RTN1C and RTN4B were identified as proteins interacting with Bcl-X_L, a member of the Bcl2 family with anti-apoptotic activity. Conversely, RTN4B is able to interact with another apoptosis inhibitor, Bcl2. Thus both the subcellular localization, and consequently the activity of these anti-apoptotic factors are regulated by reticulons. In fact, RTN1C and RTN4A have been observed to reduce the anti-apoptotic ability of their

respective interactors in mammal cells [34]. However, the ectopic expression of this RTN family member triggers extensive apoptosis in CGL4 and SaOS-2 cancer cell lines. Moreover, NogoB mRNA expression is abrogated in small cell lung cancer, suggesting a potential involvement in cancer development [35]. Additional studies indicate that NogoB overexpression causes ER stress-mediated apoptosis accompanied by ER luminal Ca²⁺ depletion [36]. On the other hand, these results contrast with other data showing any sensitivity to apoptosis induced by chemotherapeutic drugs, like staurosporine or tunicamycin, in SaOS and CHO cell lines characterized by the stable NogoB overexpression [37]. This putative NogoB antiapoptotic function is also supported by the fact that this reticulon is cleaved by caspase-7 target during apoptosis upon dephosphorylation of its Ser¹⁶ [38], suggesting a highly regulated mechanism for NogoB proteolysis. On the basis of this evidence, it is tempting to speculate that NogoB processing might contribute to apoptosis phenotype by affecting the interactions with Bcl2 and Bcl-X_L. It would be interesting to investigate whether the other RTNs are also targets for caspases as well as RTN4B/NogoB.

We have demonstrated a role for RTN1C in the modulation of ER stress-induced apoptosis. In particular, RTN1C-overexpressing neuroblastoma cells show a significant increase in their susceptibility to cell death upon ER stress induction [39]. Moreover, RTN1C overexpression induces *per se* apoptosis via ER stress induction characterized by a significant and toxic ER store calcium depletion [40]. Similarly to RTN1C, RTN3 overexpression also causes apoptosis accompanied by depletion of ER Ca²⁺ stores [41]. However, it is important to note that controversial results have been reported about the pro-apoptotic role of RTN3. In fact, it has been shown that ER stress-mediated RTN3 upregulation can be protective against several insults, contributing to the Bcl2 translocation to the mitochondria [42]. The molecular mechanisms by which RTN1C and RTN3 affect the intracellular Ca²⁺ homeostasis still remain elusive. In the past Oertle and Schwab hypothesized that RTN oligomerization could lead to the formation of pores in the ER [13]. This hypothesis could explain the intrinsic ability of RTNs to induce the Ca²⁺ efflux from ER, since no interactions of RTN1C or other RTNs involved in the ER Ca²⁺ depletion with the inositol trisphosphate (IP₃) receptors or sarcolemmal/endoplasmic reticulum Ca²⁺ pump (SERCA) have been demonstrated. It is well known that other proapoptotic Bcl2 family members, like Bak and Bax, are also localized in ER membrane where they may trigger ER-stress induced apoptosis [43], by regulating the Ca²⁺ efflux through type I IP₃ receptor [44]. Based on these findings it is tempting to hypothesize that the reticulons might induce Ca²⁺ depletion from ER by indirectly activating Bax/Bak molecules or alternatively by reducing their inhibitory interactions with Bcl2/Bcl-X_L.

It is noteworthy that the second hydrophobic domain at NogoB C-terminus is important for apoptotic function of this protein [35]. As described above the different RTN isoforms share a C-terminal reticulon homology domain containing two hydrophobic segments and a 66-amino acid hydrophilic loop. We have recently identified in the C-terminal region of RTN1C, a unique consensus sequence (GAKRH) showing 100% identity with the DNA-binding domain of histone H4 (Fig. 1). Interestingly, we showed that this sequence is essential for RTN1C-mediated ER-stress induced apoptosis, although it does not affect its ER-localization [45]. It is noteworthy that the lysine 204 present in this region is post-

translationally modified by acetylation and that this event is associated with a significant decrease in histone deacetylase activity contributing RTN1C binding to DNA. These data demonstrate a molecular mechanism by which RTN-1C might control apoptosis.

Chen et al. recently showed that RTN3 overexpression enhances apoptosis in N2a mouse neuroblastoma cells treated with an ER stress inducing drug. Interestingly, in this work an important link between reticulons and autophagy has been shown, the latter being the most important degradative pathway of the lysosomal system for the removal of cellular organelles and protein aggregates that are too large to be delivered to the proteasome [46]. They demonstrated that RTN3 plays an important role in autophagy induced by disease-associated prion proteins; in particular, the knockout of RTN3 protein by increasing autophagy induction in human prion-overexpressing N2a cells and enhances the clearance of the prion aggregates. The authors found that RTN3 inhibits beclin-1-dependent autophagy by promoting the formation of the inactive Bcl2–Beclin-1 complex [47]. Despite the molecular mechanism by which RTN3 promotes the interaction between Bcl2 and Beclin-1 is unknown, previous findings showed that RTN3 is able to bind Bcl2 and that RTNs act in the transport of constituents from ER to other membrane compartments, thus it is reasonable to believe that RTN3 could promote Bcl2 mobilization upon ER stress induction to regulate bcl2-dependent cellular processes such as autophagy or apoptosis. Based on these findings it will be interesting to investigate whether other RTNs interacting with Bcl2 are implicated in autophagy and whether their action is associated to their capacity to mobilize Ca^{2+} from the ER. In keeping with this hypothesis, the release of Ca^{2+} from ER has been recently postulated to be an important early regulatory event during autophagy induction [48].

Conclusions

Several recent studies have shown the importance of RTNs in ER homeostasis. In particular, their proapoptotic function as a novel regulator of ER stress-mediated cell death is well characterized [39]. However, the molecular mechanism by which the reticulon family members orchestrate different cellular responses is still poorly understood. Future studies should define the molecular details of how the RTN action is translated at ER level into a death stimulus. The ability to respond to perturbations in ER function is a key property of all cells. In fact, in settings of chronic ER stress, the associated apoptosis may contribute to pathophysiological processes involved in a number of prevalent diseases, including neurodegenerative diseases, diabetes, atherosclerosis and renal disease [32]. By using microarray analysis of the whole human genome, we have recently demonstrated that RTN-1C is able to specifically regulate gene expression, modulating transcript clusters which have been implicated in the onset of neurodegenerative disorders. Interestingly, we show that some of the identified genes were also modulated in vivo in a brain-specific mouse model overexpressing RTN-1C [49].

In keeping with this, the reticulons may act as modulator of PDI subcellular distribution. PDI is an ER resident protein fundamental for correct protein maturations. Therefore they act as important cellular defense against misfolded protein accumulations, typical

of neurodegenerative disorders. Noteworthy, this property has been associated with a neuroprotective effect in ALS mice models of amyotrophic lateral sclerosis (ALS) [50], and thus reticulon-mediated ER changes, including PDI relocalization, could represent a potential therapeutic target in ALS. In line with these findings, RTNs have been shown to play an important role in the nervous system both in normal and pathological settings. In fact, studies carried out on post-mortem brain tissues have revealed changes in reticulons expression in the temporal and frontal cortex of patients with Alzheimer's disease and schizophrenia [24]. In keeping with these data, human association studies have implicated mutations in RTN4 in the development of schizophrenia [51] (Fig. 2). Taken together, all these new findings provide a basis for further investigation of RTNs as a potential molecular target for use in therapy and as a specific marker for neurological diseases.

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