REVIEW

Nucleolin, a major conserved multifunctional nucleolar phosphoprotein of proliferating cells*

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Summary
Nucleolin is the major nucleolar protein of animal, plant and yeast proliferating cells. It is enriched in the most soluble nuclear or nucleolar protein extract, containing ribonucleoproteins, from which it has been purified. It has a tripartite structure in which each domain accounts for different functions. Despite its multifunctionality, the best characterized role of nucleolin is in the primary cleavage of pre-rRNA, an early step of ribosome biogenesis. In the nucleolus of proliferating cells, nucleolin is mostly located in the dense fibrillar component, following a vectorial pattern, from the periphery of fibrillar centers outwards. This pattern is lost in quiescent cells in which nucleolin is present in low levels. Nucleolin is the most phosphorylated protein of the soluble nuclear extract. It is phosphorylated by casein kinase II and CDKA, and phosphorylation is closely associated with the role of nucleolin in proliferating cells. During mitosis, nucleolin is transported from the mother to the daughter cell nucleolus in the form of processing particles, together with pre-rRNA precursors and other nucleolar proteins. It forms part of prenucleolar bodies and plays a role in nucleologenesis. Recent studies on the nucleolin function, carried out on samples with inactivated nucleolin genes (siRNA downregulated or mutants) have evidenced that nucleolin is absolutely essential for cell proliferation, for the organization of the nucleolus and for transcription and processing of pre-rRNA. In plants, nucleolin controls the auxin responsiveness, thus being involved in the regulation of plant development.

Key words: ribosome biogenesis; pre-rRNA processing; protein kinases; cell cycle; prenucleolar bodies; auxin

*This paper is dedicated to the memory of our colleague and friend Antonio Cerdido.

CELL PROLIFERATION, CELL CYCLE AND RIBOSOME BIOGENESIS

In highly proliferative cells, e.g. tumour cells in animals or meristematic cells in plants, all the basic activities of the cell (gene expression, protein synthesis, energetic pathways, signal transduction, etc.) are affected in different degrees by the mechanisms driving cell growth in order to reach the critical size capable of allowing cell division. These functional processes take place throughout a precisely organized cell cycle, whose progression is strictly regulated at several checkpoints dependent on many different signals and inputs (Mizukami 2001, Inzé and De Veylder 2006). Cell growth requires a continuous supply of proteins, which is necessary for building new cellular materials. Since ribosomes are the cellular factories in which the mRNA code is translated into proteins, the control of ribosome biogenesis is necessarily a key element of
proliferation control. It has been repeatedly shown experimentally that the process of ribosome biogenesis is highly dependent on factors regulating cell proliferation and cell cycle progression (Bernstein et al. 2007).

Factors stimulating cell growth and division produce an increase in the rate of ribosome biogenesis, which is regulated during the cell cycle, increasing from G1 to G2, and is stopped during mitosis (Hernandez-Verdun and Roussel 2003). Ribosome biogenesis is morphologically expressed by a prominent nuclear organelle, i.e., the nucleolus, and alterations in the rate of ribosome production have been morphologically detected as changes in the nucleolar structure and size and in the distribution of its subcomponents (Risueño and Medina 1986, Sáez-Vásquez and Medina 2008).

The evolution of the nucleolus during cell cycle periods is associated with variations in nucleolar proteins (Cerdido and Medina 1995, Klein and Grummt 1999). These proteins, acting as targets for factors controlling cell proliferation and cell cycle progression, and, at the same time, as regulators of the rate of pre-rRNA transcription and/or processing at different levels, could be the link connecting cell cycle and proliferation events and regulation of ribosome biogenesis at the molecular level (Olson 1991, Medina and González-Camacho 2003).

The positive correlation between cell proliferation and the activity of pre-rRNA transcription and processing also causes alterations in the size of the nucleolus and in the distribution of the nucleolar components, correlated with modifications in the proliferative state of the cell. In fact, differences in ribosome synthesizing activity are expressed as morphologically detectable changes in the nucleolus (Shaw and Jordan 1995, Medina et al. 2000). Since, as indicated above, each period of the cell cycle is associated with a particular rate of pre-rRNA synthesis and processing, a particular structural pattern of the nucleolus can be ascribed to each interphase period. These structural changes could serve as a model for the establishment of general patterns of structure-function relationships in the nucleolus, and these nucleolar structural patterns could become markers for identifying cell cycle periods (González-Camacho and Medina 2006b).

**NUCLEOLIN PURIFICATION AND GENERAL CHARACTERIZATION**

The definition and characterization of the protein population (or the proteome) of a subcellular organelle is greatly facilitated by applying methods of subcellular fractionation. It was proposed by our laboratory (González-Camacho and Medina 2006a) that fractionation of nuclear (or nucleolar) proteins would be carried out according to the solubility of proteins in buffers of increasing ionic strength. This physical criterion, accompanied in some steps by the use of additional reagents such as detergents or enzymes, produced fractions of functional significance. The proposed procedure yields five fractions, the first of them containing proteins associated to the nuclear envelope and remnants of the cytoskeleton; the second, which is soluble in low ionic strength, is called “S2 fraction”, and contains ribonucleoproteins active in nuclear RNA metabolism; after increasing ionic strength and digesting with DNase the result is the chromatin fraction, and finally, the fourth and fifth fractions correspond to the nuclear matrix and are obtained, respectively, by solubilization in high salt concentration and in the form of the residual pellet, only soluble in 7 M urea under sonication (González-Camacho and Medina 2006a).

The soluble fraction of nuclear proteins (S2) from proliferating cells has been indeed the object of many studies which have shown that this fraction contains many proteins forming RNP complexes, especially proteins actively involved in the synthesis and processing of pre-rRNA. The major protein of this extract, either obtained from isolated nuclei or nucleoli, is the nucleolar protein nucleolin, formerly called C23 (Busch et al. 1978, Bugler et al. 1982). This finding, firstly obtained in mammalian cells, was corroborated by our laboratory in plant meristematic cells (De Cárcer et al. 1997). Moreover, nucleolin is the most abundant non-ribosomal protein, not only in the soluble S2 extract, but in the nucleolus of proliferating cells (Lapeyre et al. 1987, Martín et al. 1992).

The structure of nucleolin, as described in animals, plants and yeast, is composed of three domains: a) a highly charged acidic stretch at the amino terminus with characteristic repeats; b) a central domain containing four (animal) or two (plant and yeast) RNA Recognition Motifs (RRMs), each of which contains a highly conserved RiboNucleoProtein-1 (RNP-1) octamer motif and a less conserved RNP-2 hexamer motif, and c) a conserved Gly- and Arg-rich carboxy-terminal sequence, called the GAR domain (Ginisty et al. 1999).

The complex multipartite structure of nucleolin reflects its multifunctional role: the acidic N-terminal region, is involved in the interaction with components of the pre-rRNA processing complex and also in the...
control of rDNA transcription (Roger et al. 2003): the central region has been implicated in RNA binding specificity and affinity for pre-rRNA sequences (Serin et al. 1996), and the C-terminal region or GAR domain interacts with several ribosomal proteins (Bouvet et al. 1998) and also binds RNA, but in a nonspecific manner and with low affinity (Ghisolfi et al. 1992).

The role played by nucleolin in the primary cleavage of pre-rRNA, occurring in the 5’ external transcribed spacer (5’ETS), is particularly well known in mammals and yeast, involving the formation of a snoRNP complex in which the nucleolar protein fibrillarin and several snoRNAs have been identified (Kondo and Inouye 1992, Ginisty et al. 2000). In plants, a similar snoRNP complex was purified and characterized, in which the nucleolar proteins nucleolin and fibrilalin, as well as the snoRNAs U3 and U14 were identified, and which was shown to bind to 5’ETS RNA and accurately cleaving it at a cleavage site mapped in vivo (Sáez-Vásquez et al. 2004).

Although nucleolin has been involved essentially in ribosome biogenesis, it has also been implicated in a number of additional processes that take place in the nucleus and in the cytoplasm, including RNA pol II transcription regulation, DNA replication, mRNA stability /translation and assembly of RNP complexes (see Sáez-Vásquez and Medina 2008).

LOCALIZATION IN SITU OF NUCLEOLIN AND RELATION TO CELL PROLIFERATION

Nucleolin has been localized in situ at the ultrastructural level within the nucleolus in animal and plant cells, by using anti-nucleolin antibodies and the immunogold method (Escande et al. 1985, Martin et al. 1992, De Cárcer et al. 1997). The adscription of nucleolin to one or more of the different nucleolar subdomains has a physiological significance, since the correlation of these structural subnucleolar domains with the different steps of pre-rRNA synthesis and processing is well established. In onion proliferating cells the quantification of immunogold labelling resulted in a preferential localization of the protein in the dense fibrillar component (DFC) (Martin et al. 1992, De Cárcer et al. 1997). Moreover, we examined in detail the distribution of labelling showing that the DFC was not evenly labelled, but particles accumulated in the neighborhoods of fibrillar centres (FCs). Most FCs did not contain gold particles in their interior (Fig. 1, A1 and A2). The quantitative evaluation of the labelling distribution, by means of the Radial Distribution Function confirmed this estimation (not shown). Therefore, nucleolin has a vectorial distribution in the nucleolus, from the border of FCs outwards.

In quiescent cells, cell cycle progression and ribosome biogenesis are arrested, nucleoli do not contain any granular component and FCs are always large, low in number and heterogeneous in structure, containing condensed chromatin inclusions (Risueño and Moreno Díaz de la Espina 1979). In these cells, we showed that the vectorial distribution of nucleolin was lost and labelling was dispersedly located throughout the whole nucleolar body (Fig. 1B). Quantitative analysis confirmed this observation.

The quantification of the level of labelling showed almost three times more labelling in proliferating than in quiescent cells (Fig. 1C) and was indicative of the relationship of nucleolin with cell proliferation.

Furthermore, the in situ localization is consistent with the implication of nucleolin in transcription of the ribosomal genes and also in the early processing of the pre-rRNA transcript. Transcription was shown to take place around FCs and in the transition zone between FCs and the DFC (Martin and Medina 1991, Shaw and Jordan 1995, De Cárcer and Medina 1999); in this zone, nucleolin has been shown to colocalize with a protein immunologically related to the transcription factor UBF in onion cells (De Cárcer and Medina 1999). Early pre-rRNA processing has been described as taking place in the region of the DFC closer to FCs (Shaw and Jordan 1995, De Cárcer and Medina 1999), which contains high levels of nucleolin. The presence of nucleolin in quiescent cells, even at lower levels than those of proliferating cells, can be related to the existence of a fraction of the protein which is insoluble and, consequently, is associated to the nuclear matrix (Mínguez and Moreno Díaz de la Espina 1996) (see next section). It is not known whether the functional role of this protein in these cells is merely structural, or represents a transient storage form that can be eventually activated when cellular metabolism is resumed.

Otherwise, a correlation of increased nucleolin expression with cell proliferation and cell cycle progression has been directly demonstrated in peas (Reichler et al. 2001) and also in alfalfa (Bögre et al. 1996). In onion cells, variations of the nucleolin levels throughout the cell cycle have been investigated, and the highest levels have been found in G2, the period characterized by the highest rate of pre-rRNA synthesis and processing (González-Camacho and Medina 2006a).
NUCLEOLIN PHOSPHORYLATION

One of the most important features of nucleolin is phosphorylation. Actually, the dynamics of phosphorylation-dephosphorylation largely determine most of the functions of the protein.

We performed a pseudo-in vivo phosphorylation test by endogenous kinases present in the nuclei by incubating isolated onion cell nuclei, with radioactive orthophosphate $[^{32}\text{P}]$. Nuclei were then fractionated and the S2 protein extract was electrophoretically separated by SDS-PAGE and subjected to autoradiography (Fig. 2A). The phosphoprotein pattern obtained showed that the band migrating at 100 kDa, corresponding to nucleolin, was the most phosphorylated band of the extract (Fig. 2A).

Several potential phosphorylation sites by specific kinases have been identified in the sequences of nucleolin both in mammals (Lapeyre et al. 1987) and in plants (Sáez-Vásquez J., personal communication). In mammals, it was demonstrated that two kinases, namely cdc2 kinase (or CDKA) and casein kinase II (CKII), known to play crucial roles in the regulation and coordination of cellular events involved in cell cycle and proliferation, are capable of phosphorylating nucleolin (Caizergues-Ferrer et al. 1987, Belenguer et al. 1990).

In onion cells, we tested the exogenous in vitro phosphorylation by CKII. When it was performed on the S2 nuclear protein extract, nucleolin (100 kDa) was the most phosphorylated band, as visualized by autoradiography (Fig. 2B). In addition, purified

Fig. 1. Subnucleolar distribution of nucleolin after immunogold electron microscopy in onion proliferating (A1, A2) and quiescent (B) root meristematic cells, quantitatively assessed. (A1, A2): Electron micrographs showing the ultrastructural localization of nucleolin in proliferating cells. A2 is an enlargement of the area outlined in A1. Labelling was concentrated in the dense fibrillar component (DFC) surrounding fibrillar centers (arrows). (B): Ultrastructural localization of nucleolin in onion root quiescent cells. Labelling was distributed throughout the nucleolus, which is much smaller than in proliferating cells and is exclusively formed by dense fibrillar component (DFC) and fibrillar centers (arrows), being absent the granular component.
nucleolin became heavily phosphorylated in vitro by this kinase (Fig. 2B). The additional bands appearing in the test on purified nucleolin are due to CKII self-phosphorylation, as shown in a control test performed with purified CKII (Fig. 2B).

In order to estimate the relationship of nucleolin phosphorylation to cell proliferation, we evaluated the phosphorylation state of nucleolin by an experiment of in vitro dephosphorylation with alkaline phosphatase of the S2 nuclear extract obtained from onion root meristematic cells. Bands separated by SDS-PAGE from either treated or untreated extracts were visualized by Western blotting using an anti-nucleolin antibody. Dephosphorylation resulted in the loss of the 100 kDa band and its substitution by a 90 kDa band, as well as in the enhancement of a band of 64 kDa (Fig. 2C). Interestingly, these two bands were the major bands revealed by the antibody on intact nuclear matrix fractions obtained from the same cells (Fig. 2C). This strongly suggests that insoluble nucleolin, associated with the nucleolar matrix, is in an unphosphorylated state. As mentioned above, this insoluble form of nucleolin has a structural role and/or is a storage form of a transitory inactive nucleolin.

Furthermore, bi-dimensional Western blotting with an anti-nucleolin antibody revealed 17 spots at the level of 100 kDa in the S2 extract of meristematic cells (González-Camacho and Medina 2004). All the spots detected form a cluster through a pI range of 4.3–6.6. This cluster gives an account of different states of phosphorylation exhibited by the protein in dependence on the nucleolar activity and the cell cycle phases. In contrast, only 8 spots were found in the extract from non-meristematic nuclei, whose pI range was shortened to 4.8–6.1. This indicates a substantially lower amount of phosphorylation variants, associated with the drop of the proliferation capacity and of the nucleolar activity (González-Camacho and Medina 2004).

NUCLEOLIN IN MITOSIS

Nucleolin is essential for the normal course of mitosis, including chromosome congression in metaphase (Ma et al. 2007). The localization of nucleolin during mitosis, studied by immunofluorescence in onion cells, showed a pair of
Immunofluorescent localization of nucleolin during mitosis in squashed onion root meristematic cells. (A, C, E): nucleolin; (B, D, F): DAPI counterstaining of DNA. (A, B): Metaphase. The nucleolar organizer (NOR; arrow) was labelled. (C, D): Anaphase. Labelling appeared among chromosomes, with the shape of a perichromosomal sheath. (E, F): Telophase. Prenucleolar bodies of different sizes were all labelled by the antibody. (G): Telophase, double-labelled by anti-nucleolin antibody (green), and anti-NOR90 human autoantiserum (red), detecting an rDNA-binding protein immunologically related to the transcription factor UBF. The picture is a merged image in which colocalization zones appear in yellow. All prenucleolar bodies contained nucleolin, but only a few of them were also labelled by anti-NOR90 (arrowheads point to colocalization; arrows point to exclusive labelling by anti-NopA100). Prenucleolar bodies containing both epitopes were few and larger, and they were located at distal positions with respect to poles.

Bright spots in metaphase, presumably corresponding to the chromosomal nucleolar organizer (NOR) (Fig. 3A, B). In anaphase, labelling is observed at the periphery of chromosomes, in the form of a perichromosomal sheath. In some cases, it was observed that the material of the perichromosomal sheath condensed to form rounded structures, resembling prenucleolar bodies (PNBs). Furthermore, many small bright dots were seen in the cytoplasm, outside the spindle (Fig. 3C, D). Finally, in telophase, all the material revealed by the anti-nucleolin antibody appeared either inside the reorganizing nuclei, as PNBs very different in size to one another, or in the cytoplasm, as small dots (Fig. 3E, F, G).

This localization of nucleolin during mitosis is shared with other proteins involved in pre-rRNA processing (fibrillarin, B23), and also with pre-rRNA processing intermediates, which were synthesized in the preceding G2. The presence of nucleolin is critical for the assembly of these proteins and RNAs forming the nucleolar processing complex particles called “processomes” (Ma et al. 2007). These particles are dispersed from the mother cell nucleolus in prophase and are carried to the daughter cells by chromosomes in the form of a perichromosomal sheath, which is concentrated in PNBs at telophase, which contribute to nucleologenesis, i.e. the formation of the new nucleolus in daughter cells (Medina et al. 1995, Dundr et al. 2000, Ma et al. 2007). Therefore, PNBs are not structures which originate “de novo”, but they are formed from the materials contained in the perichromosomal sheath which, in turn, are formed from the dispersed remnants of the mother cell nucleolus.

Immunolocalization of nucleolin in telophase was combined with the detection of the antigen of the human autoantiserum NOR90 (Fig. 3G). This serum recognizes in mammalian cells the RNA polymerase I transcription factor UBF (Chan et al. 1991) and it has been shown in plant cells to recognize a homologous epitope (Rodrigo et al. 1992), exclusively nucleolar in localization (Rodrigo et al. 1992, De Cárcer and Medina 1999). Although no obvious UBF homolog has been found in plants, this epitope most probably contains the HMG domain and, therefore, has an rDNA-binding capacity. Interestingly, the colocalization experiment revealed two types of PNBs: those of the first type, containing the two antigens, were few (two or three per nucleus); larger, and appeared located at distal positions with respect to the poles of the mother cell. The second type of PNBs was smaller, more numerous, randomly located in the reorganizing nucleus and was only labelled by an anti-nucleolin antibody (Fig. 3G). The existence of two types of PNBs, depending on
whether rDNA-bound elements of the transcription complex are present or not, was first shown using RNA polymerase I as the marker for the transcription complex (Jiménez-García et al. 1989). Since pre-rRNA synthesis is resumed after mitosis in the NOR, where the materials of PNBs, including incompletely processed pre-rRNA molecules and nucleolar proteins, are recruited (Dundr et al. 2000, Medina et al. 2000) the few large UBF-containing PNBs labelled by the anti-NOR90 serum are, in fact, reorganizing nucleoli. The same interpretation can be attributed to PNBs containing RNA polymerase I (Jiménez-García et al. 1989). This association of nucleolin with rDNA in reorganizing nucleoli is essential for nucleologenesis because RNA-interference-mediated nucleolin-depleted cells are unable to reorganize the nucleolus, which remains disorganized and partially dispersed through interphase (Ma et al. 2007).

At the time of nucleologenesis, a flow of materials is established between PNBs and the NOR, so that PNBs become progressively smaller whereas the new nucleolus emerges and grows at the site of the NOR (Savino et al. 2001). Interestingly, nucleolar protein recruitment at the NOR occurs sequentially, the early processing proteins being recruited first on transcription sites, while later processing proteins remain in PNBs for a longer time, before migrating to the NOR (Angelier et al. 2005).

**ASSESSMENT OF THE FUNCTIONAL ROLE OF NUCLEOLIN IN RIBOSOME BIOGENESIS BY MEANS OF THE INACTIVATION OF THE NUCLEOLIN GENE**

A remarkable advance regarding the function of nucleolin comes from recent studies carried out in both animal and plant cells using the downregulation of the expression of the nucleolin gene, either by the RNA-interference (siRNA)-mediated depletion, or by mutants containing disrupted nucleolin genes. In the preceding paragraph it was mentioned that siRNA-downregulated nucleolin expression increased our understanding of the role of nucleolin during mitosis, particularly regarding the proper formation of the metaphase plate and the nucleolus reorganization in telophase (Ma et al. 2007).

The same approach used by another research team on mammalian cultured cells (Ugrinova et al. 2007), together with a more recent study based on the generation of a nucleolin conditional knockout (Storck et al. 2009) showed a rapid and drastic drop of the cell proliferation rate associated with the decrease in the levels of nucleolin; this drop was due to the arrest of cell cycle progression, either in G1 or in G2, accompanied by the entry in apoptosis of a significant number of cells; furthermore, the nucleolar substructure was re-arranged, appearing with the components segregated, resembling the structure shown after RNA polymerase I transcription inhibition by actinomycin D treatment. In fact, the loss of function of nucleolin caused a drastic reduction in rRNA gene transcription and in contrast, pre-rRNA processing was shown to be only slightly affected.

In plants, the analysis of three independent Arabidopsis nucleolin mutants, all of them encompassing a disruption of the nucleolin gene, showed that the lack of expression of nucleolin phenotypically resulted in a reduced growth rate, a prolonged life cycle, pointated leaves, a defective vascular pattern and aberrant leaf venation (Kojima et al. 2007, Petricka and Nelson 2007, Pontvianne et al. 2007). Therefore, nucleolin plays a major role in plant growth and development and even has a considerable influence on the structure of the plant. Most probably, these effects at the level of the whole plant are the consequence of the alteration of ribosome biogenesis, but no direct evidence has been reported for any of the phenotypic effects.

At the molecular level, independent analysis of the three nucleolin mutants has consistently revealed that the absence of the protein induces changes in pre-rRNA processing, especially in the early cleavage that occurs at the level of the 5'ETS (Kojima et al. 2007, Petricka and Nelson 2007, Pontvianne et al. 2007). Intermediate pre-ribosomal precursors, and particularly the 35 S pre-rRNA (Petricka and Nelson 2007) appear to be accumulated in the mutants in relation to the wild type. This is associated with our finding that the nucleolus of mutant plant cells contains the so-called nucleolar perichromatin-like granules (Pontvianne et al. 2007), structures characterized to as containing wrongly processed pre-rRNA.

The molecular alterations in the nucleolin mutants lead to nucleolar disorganization, involving the loss of the spherical shape of the nucleolus and the hard discrimination of nucleolar subcomponents, being substituted by a loose accumulation of fibrils and granules (Pontvianne et al. 2007).

Plants, in contrast to animals, contain at least two genes encoding nucleolin or nucleolin-like proteins, called AtNUC-L1 and AtNUC-L2 (Pontvianne et al. 2007). In Atnucl1 mutant plants, the homologous AtNUC-L2 gene, which is usually repressed under normal growing conditions, is induced to be expressed. We have found that AtNUC-L2 localizes
in the nucleolus of Atmuc-L1 and might rescue, at least partially, the loss of AtNUC-L1 (Pontvianne et al. 2007).

NUCLEOLIN AND AUXIN IN PLANTS

Nucleolin loss of function in plants alters the auxin responsiveness with regard to both auxin transport and auxin response. It was suggested that nucleolin might be involved in auxin-dependent control of organ growth and patterning, potentially indicating a correlation between auxin regulation and ribosome biogenesis (Petricka and Nelson 2007). Eventually, this could be seen as a way in which nucleolin controls plant development, since the phytohormone auxin influences multiple aspects of growth and differentiation in plants. Low levels of auxin induce cell elongation, enlargement and differentiation, whereas high levels of auxin stimulate cell proliferation and cell cycle progression (Magyar et al. 2005).

Levels of nucleolin have been found to be depleted in plant meristematic cells grown during spaceflight, i.e. under environmental conditions of weightlessness or microgravity; this was associated with the uncoupling of cell growth and proliferation in these cells (Matía et al. 2010). At the same time, growth in microgravity results in a substantial inhibition of the auxin polar transport (Ueda et al. 1999). This strongly suggests that the role of auxin in the maintenance of the coupling of cell growth and proliferation under normal gravity conditions is lost, at least partially, as a consequence of the change in environmental gravity (Medina and Herranz 2010). Most likely, nucleolin plays a part in this alteration.

FUTURE PROSPECTS

Despite this intensive and extensive work, many points remain obscure on our understanding of the functional role of nucleolin in ribosome biogenesis and in other cellular functions, related or not to what has been considered up till now as the main role of the protein; namely its participation in the synthesis of ribosomes. Even, the highly productive and promising method of using either mutants or siRNA to block the expression of the nucleolin gene is still at the beginning of its course. These are our next challenges in the investigation of this exciting and crucial protein.

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