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SHORT COMMUNICATION

Extra-Ribosomal Functions of the Ribosomal Protein, RPS3 as Predicted by *In Silico* Analysis

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ABSTRACT

Products of ribosomal protein (RP) genes have been found to play extra-ribosomal roles that range from DNA repair to RNA splicing. Their association with congenital disorders or cancers has also been widely documented. However, the relatively large number of different RPs, each with perhaps unique biological roles, has compounded the comprehensive elucidation of the physiological functions of each RPs. Experimental functional studies on the many and variegated RPs are labour intensive, time-consuming and costly. Moreover, experimental studies unguided by theoretically insights entail inaccurate results. Therefore, knowledge on the actual roles of these proteins remains largely undefined. A valid alternative is the use of bioinformatics resources to computationally predict functional roles of these biomolecules. Findings from such *in silico* studies of the RPS3 are reported herein. We reveal an array of possible extra-ribosomal functions that includes regulation of transcription (including via NF-κB-mediated, POK-induced and DNA-dependent), regulation of p53 activities and its stabilisation, inflammatory immune response, modulation of nNOS activities, and anti-oxidative capabilities. Our findings provide computational prediction of *de novo* extra-ribosomal functions of RPS3. These results will enhance the theoretical basis for designing future experimental studies on elucidating its definitive physiological roles.

Keywords: Protein models, RPS3, structural neighbours

The classical understanding of ribosomal proteins (RPs) is often confined to their functions as essential components of the ribosomes important for cellular ribosome-mediated protein synthesis. Nevertheless, since the mid-90s, their roles beyond ribosome-mediated protein biosynthesis (extra-ribosomal roles) such as association with cellular development, congenital diseases, and even cancers have been described (Wool, 1996; Noara, 1999). For example, *RPS4* and *RPL6* have been linked to Turner and Noonan syndromes respectively (Fisher *et al*., 1990; Kenmochi *et al*., 2000). In fact, many of the cancer-related studies demonstrated dysregulated expression of RP genes in diseased cases. Single and multiple *RP* genes were found to be over-expressed in leukaemic and solid tumours cells (Bassoe *et al*., 1998; Ruggero & Pandolfi, 2003), and in nasopharyngeal carcinoma cells (Sim *et al*., 2010). Aberrant expressions of RP genes have been linked to a wide range of cancer-types including carcinomas of colorectum (Pogue-Geile *et al*., 1991; Kasai *et al*., 2003; Sim *et al*., 2006), breast (Henry *et al*., 1993), prostate (Vaarala *et al*., 1998), uterine cervix (Cheng *et al*., 2002), esophagus (Wang *et al*., 2001), liver (Kim *et al*., 2004), nasopharynx (Sim *et al*., 2008) and in glioblastoma and multiform brain tumours (Lopez *et al*., 2002).

Despite the widely documented association between deregulated expression of RP genes and cancers, conclusive understanding on the definitive functional roles of these genes in organogenesis and oncogenesis is unclear. In the case of *RPS3*, even though higher expression levels were observed in tissues of colon adenocarcinomas and adenomatous polyps compared to those of adjacent normal colonic mucosa (Pogue-Geile *et al*., 1991) their actual roles in colorectal tumourigenesis hitherto are not fully understood. Access to information on extra-ribosomal functions of the product encoded by *RPS3* and other RP genes

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is hindered by the fact that existing experimental studies focus mainly on a comparative analysis of the expression patterns of RP genes in diseased and normal cases. Such type of knowledge neither fully and properly defines the physiological significance of RP genes nor delineates the biochemical pathways mediated by their protein products. No doubt, experimental studies such as functional assays can be utilised for this purpose. However, these methods are often technically-demanding, time-consuming, and require high cost equipment. Functional assays that are unsupported by valid theoretical consideration would therefore be a risky and profligate approach. A rational solution to this is the use of biocomputing approaches to gain conceptual insights into the physiological roles of biomolecules prior to the design of experimental studies. The basis of *in silico* analysis in this context hinges on the concept that the function of a protein is tightly linked to its three dimensional (3D) structure (Peitsch, 2002). This form of analysis is feasible via Biocomputing tools amenable for predicting protein functions based on the information of their (proteins) sequences, structures, evolutionary patterns and known associations with other proteins (Punta & Ofran, 2008). In fact, computational extrapolation of protein functions based on the sequence-to-structure-to-function paradigm is already achievable via a single platform that consolidates various tools (Roy *et al*., 2010). More importantly, functional extrapolations of rational 3D protein models can often be corroborated with their physiological significance as inferred from experimental discoveries. For example, the constructed 3D model of the S1 domain of SARS-associated coronavirus (SARS-CoV) spike protein exhibits structural similarity to the influenza virus neuraminidase protein, suggesting similar roles between anti-influenza virus inhibitors and anti-neuraminidase antibody, of which was subsequently verified in experimental studies on anti-SARS antibody functions (Zhang & Yap, 2004). The automated prediction of ribosomal protein functions via the sequence-to-structure-to-function paradigm coupled with simulated docking analysis is not unprecedented, and has been carried out for RPL27 and RPL37a (Chan & Sim, 2013).

Herein, we report findings on computational derivation of 3D model of RPS3, which in turn, were used to predict more of its extra-ribosomal functions. The former effort was carried out via comparative homology modelling using the 3D-JIGSAW server (http://www.bmm.icnet.uk/~3djigsaw/) and the latter approach was conducted using the strategy of structural neighbour prediction and function-matching via the Vector Alignment Search Tool (VAST) server (http://www.ncbi.nlm.nih.gov/Structure/VAST/vast.shtml). For analysis using 3D-JIGSAW platform, generation of 3D protein model is based on the similarity to homologues of known structures (Bates *et al*., 2001). The 3D-JIGSAW server optimises this search for candidate templates by incorporating the Domain Fishing scheme of combining database of protein structures PBD, the protein families database PFAM, and the structural classification of proteins SCOP (Contreras-Moreira & Bates, 2002). In principle, this is done by logically dividing the nascent templates from PDB, using PFAM and SCOP to obtain queries of single domains, upon which allows templates of remote homologies to be added into the total profile of the initial query (Contreras-Moreira & Bates, 2002). Hence, a more accurate comparative homology modelling strategy can be implemented. On the other hand, the VAST analysis involves a similarity-search algorithm that searches against medium-redundancy subset of PDB data to align structures of query proteins with their corresponding structural neighbours (Gibrat *et al*., 1996). Basically, VAST program allows systematic structure-structure search and alignment between 3D structures of query and proteins in the Molecular Modelling Database (MMDB) in order to identify statistically significant similarities – a process of structural comparison that is based on purely geometric criteria (Gibrat *et al*., 1996).

In the first part of our analysis, the sequence of human RPS3 was obtained from the GenBank database (Accession no. AAB19349; via the National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/). This amino

acid sequence was submitted to a 3D-JIDSAW website in FASTA format, and allowed for automatic alignment. The result was returned in the form of pdb-formatted file via e-mail. Constructed logical 3D model of RPS3 was then viewed using the RasMol software (Version 2.7.4.2). Secondly, functional (extra-ribosomal) prediction of RPS3 was based on identifying structural neighbours of the RP, and then, using known functions of structural neighbours to derive functions. To search for structural neighbours, constructed 3D model of RPS3 was submitted as Protein Data Bank (PDB) files to the VAST server. Significance of the comparison is represented by the *p*-value where 0.001 indicates that the odd of a match by pure chance is 1/1000. Files of the structural comparison were downloaded and viewed by Cn3D software (http://www.ncbi.nlm.nih.gov/Structure/CN3D / cn3d.shtml). The annotated databases of PDB (http://www.rcsb.org/pdb/home/ home.do), PFAM (http://pfam.sanger.ac.uk; Finn *et al*., 2008), and SCOP (http://scop.mrc-lmb.cam.ac.uk/scop; Murzin *et al*., 1995) were employed for functional annotation of the proteins for the purpose of functional derivation.

Our constructed rational 3D model of RPS3 shows the presence of two domains, Domain 1 and 2 (Figure 1A). Domain 1 has 4 α-helices, and a three-stranded anti-parallel β-sheet that has an additional short part comprising only one amino acid residue (Val). Domain 2 has 5 α-helices, and 4-stranded anti-parallel β-sheet with an additional short part comprising only one amino acid residue (Met). In comparison to the recently reported crystal structure of yeast RPS3 (Holzer *et al.*, 2013), there is consistency in the presence of the double domain feature, and this validates our logical model. Similar to first domain of yeast RPS3, Domain 1 of human RPS3 harbours the K-homology (KH) motif. Known functions of this KH domain tally with some of the known extra-ribosomal roles of RPS3 (Table 1), hence validating our *in silico* approach. These include involvement in the NF-κB transcription factor activity as a subunit of the NF-κB complexes (Wan *et al*., 2007) and interaction with p53 and MDM2 as a component in the p53/MDM2 regulatory loop (Yadavilli *et al*., 2009). Structural neighbours of RPS3 Domain 1 are the murine macrophage migration inhibitory factor (MIF) and BolA1 protein (Figure 1B). The amino acid sequence similarity between human and mouse MIF is 89% (Donn & Ray, 2004), and our pairwise analysis (via Bl2Seq) revealed 81% similarity between murine and human BolA1 proteins. Murine MIF is divided into 3 domains and its first domain resembles Domain 1 of RPS3. The MIF protein, a cytokine that is released from T cells or macrophages, is normally taken up by target cells wherein it then interacts with intracellular signalling molecules to inhibit p53 function (Donn & Ray, 2004). From this, we infer that a possible extra-ribosomal role of RPS3 could be the inhibition of p53 function. Our data not only affirm the involvement of RPS3 in the modulation of p53 activity, but further implied a RPS3-mediated inhibition of p53 function via interaction with intracellular signalling molecules. Although the associated signalling factors and interaction mechanism remain to be researched further, our *in silico* findings added new theoretical insight into the regulation of p53 by RPS3.

The hBolA1 has a DNA-binding motif and was demonstrated to be capable of being secreted, and is suggested to be non-classical secreted protein (Zhou *et al*., 2008). The structural similarity between RPS3 Domain 1 with BolA1 implies that RPS3 may be a secreted DNA-binding protein capable of transcription regulation.

Domain 2 of RPS3 is structurally similar to the neuronal nitric oxide synthase inhibitory protein, PIN (protein inhibitor of nNOS) (Figure 1C). In this case, RPS3 Domain 2 shares similar basic structures of two α-helices and one four-stranded β-sheet with Domain 1 of PIN, which contains the Dynein Light Chain 1 domain. Expression of NOS correlates positively with tumour grade in human astrocytic tumours (Cobbs *et al*., 2005), and NOS inhibitors reduce local tumour growth in mouse models that have sarcoma-derived tumours or malignant melanoma (Cahlin *et al*., 2000). Studies have shown that RPS3 exhibit differential distribution in brain tissues, particularly in

neurons of the ependymal cells, hippocampus and stantia nigra pars compacta regions (Choi *et al*., 2006). Taking together the known functions of PIN and the localisation of RPS3, we speculate that RPS3 may play a role in the regulation of neuronal cell growth via modulating the activities of nNOS or nNOS-like proteins in these cell types.

Domain 2 of RPS3 also shows structural similarities to the proteins of zinc finger 295 (ZNF295) (Figure 1D), and the cocaine and amphetamine regulated transcript (CART) (Figure 1E). In the case of ZNF295, two β strands of its first domain superposed with Domain 2 of RPS3. The human ZNF295 protein belongs to the family of POK (POZ and krüppel) transcription factors that function as transcription repressors, and in concert with ZFP161 (another POK protein), it acts in the bi-directional (activation and repression) control of gene expression (Wang *et al*., 2005). The partial structural alignment between ZNF295 and RPS3 Domain 2 suggest some possibilities of the latter in activating and repressing DNA-dependent transcription via collaborating with members of the POK protein family. In the case of RPS3 and CART, structural alignment occurs between two of CART's β strands and two of the four RPS3's β strands (Figure 1E). The human CART peptides are found in the nervous and gastrointestional systems – a sort of a 'brain-gut' peptide that can act as a neurotransmitter as well as a hormone (Landerholm *et al*., 2012). These CART peptides are found to localise at the mitochondria and functions as antioxidant in cases where mitochondrial DNA, cellular proteins and lipids are exposed to hydrogen peroxide-mediated oxidative stress (Mao *et al*., 2012). Our data on the structural alignment between Domain 2 of RPS3 and CART strengthens the relevance of the former as an active protein in brain cells, and further suggests an antioxidant role.

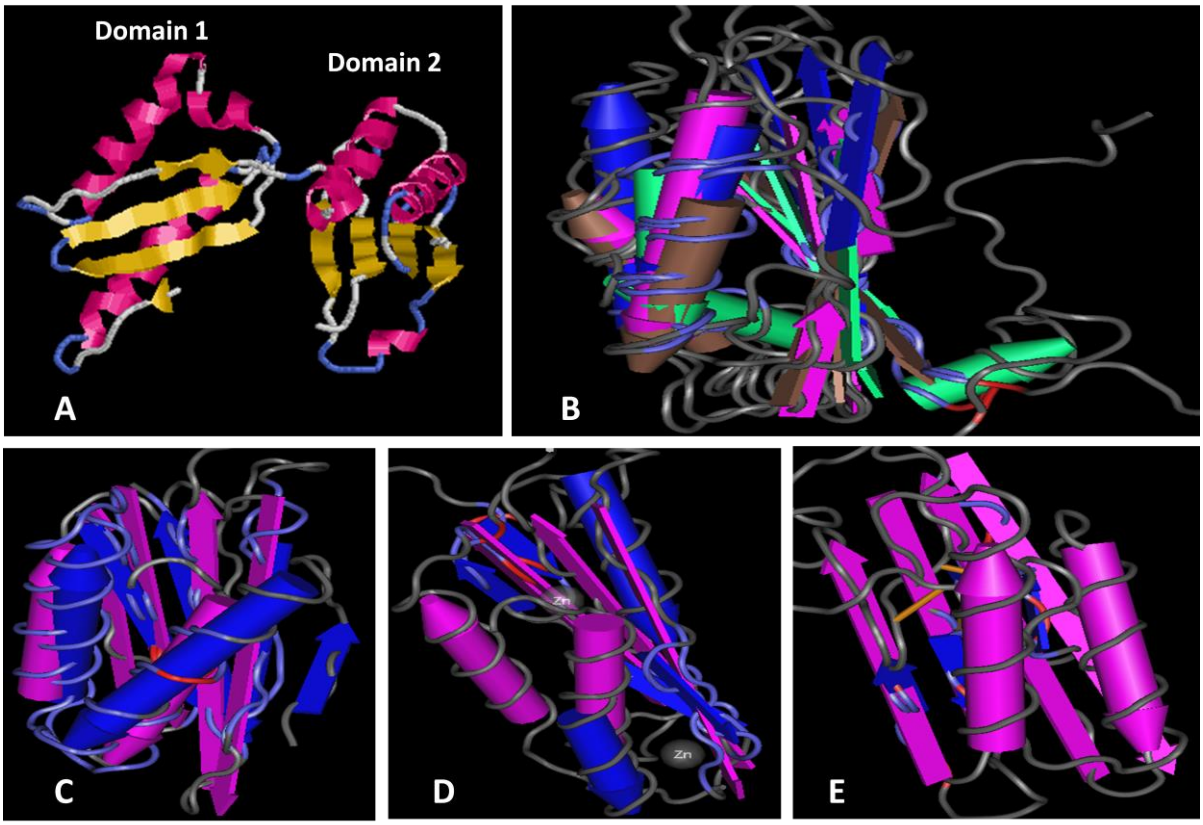

Figure 1. (A) Logical 3D structural model of RPS3 (α-helices and β-sheets are represented by red and yellow colour respectively); and three dimensional (3D) superposition of RPS3 with its structural neighbours; **(B)** Domain 1 (purple) with human KH domain (brown), murine BolA 1 protein (green), and murine macrophage migration inhibitory factor (blue); **(C)** Domain 2 with human protein inhibitor of neuronal nitric oxide synthase (blue); **(D)** Domain 2 with human zinc finger protein 295 (blue); and **(E)** Domain 2 with human cocaine and amphetamine regulated transcript protein (blue). In diagrams of **(B)** to **(E)**, the cylindrical and flat arrows represent regions of helices and strands respectively.

It should also be noted that a known function of RPS3 is the induction of apoptosis, albeit not reveal in our analysis. The 'death domain' (adjacent to KH Domain) is crucial for this function, and apoptotic induction is most probably via a caspase-dependent mechanism (Jang *et al*., 2004). More precisely, this may involve recruitment of RPS3 to the death-inducing signalling complex (DISC) by Tumour necrosis factor receptor type 1-associated DEATH domain (TRADD) protein in response to extracellular stresses (Jang *et al*., 2012). Our results of known and predicted *de novo* functions of RPS3 are summarized in Table 1.

In conclusion, our approach of using computational strategy to predict extra-ribosomal roles of RPS3 yields a list of several *de novo* functions (Table 1). This method of *in silico* analysis has never being applied on RPS3 before this, and hence our data adds new and important baseline information to guide the design of future experimental studies in elucidating the complete and definitive functions of RPS3.

Table 1. VAST results of RPS3 with structural neighbours and inferred functions, and known functions of RPS3 (based on literature).

Domain	Structural neighbours	*p*-value	Inferred functions	Known functions
1	Human KH domain (PDB ID: 1wh9_A)	0.0014	• Involvement in p53/MDM2 regulatory loop • Regulation of NF-κB transcription factor activity	Regulation of p53 (Yadavilli *et al*., 2009) Regulating NF-κB-mediated functions (Wan *et al*., 2007) Induction of apoptosis (Jang *et al*., 2004; Jang *et al*., 2012) DNA repair (Hegde *et al*., 2009) Innate immune response (Gao *et al*., 2009) Inflammatory immune response (Ahn *et al*., 2010) Translation self-regulation (Kim *et al*., 2010)
	Murine MIF (PDB ID: 2gdg_A)	0.0061	• Inhibition of p53 activities	
	Murine BolA 1 (PDB IB: 1v60)	0.0021	• DNA binding • Regulation of transcription	
2	Human PIN (PBD ID: 1cmi_A)	0.0364	• Regulation of neuron cell growth • Modulating nNOS activities	
	Human ZNF295 (PDB ID: 1wjp_A)	0.0038	• Regulation of DNA-dependent transcription • Association with POK proteins	
	Human CART (PDB ID: 1hy9_A)	0.0261	• Anti-oxidative activities in brain cells	

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