

Application of Staining Intensity Correlation Analysis to visualize Protein Colocalization at a Cellular Level

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Abstract— Mutations of the telomeric copy of the *survival motor neuron 1 (SMN1)* gene cause spinal muscular atrophy. A deletion of the *Eef1a2* gene leads to lower motor neuron degeneration in wasted mice. Indirect evidences have been shown that the eEF1A protein family may interact with SMN, and our previous study showed that abnormalities of neuromuscular junctions in wasted mice were similar to those of *Smn* mutant mice. To determine potential colocalization between SMN and tissue-specific translation elongation factor 1A2 (eEF1A2), an immunochemical analysis of HeLa cells transfected with the plasmid pcDNA3.1(+)-hEEF1A2-myc and a new quantitative test of colocalization by intensity correlation analysis (ICA) was used to explore the association of SMN and eEF1A2. Here the results showed that eEF1A2 redistributed from the cytoplasm to the nucleus in response to serum and epidermal growth factor. In the cytoplasm, compelling evidence showed that staining for myc-tagged eEF1A2 varied in synchrony with that for SMN, consistent with the formation of a SMN-eEF1A2 complex in the cytoplasm of HeLa cells. These findings suggest that eEF1A2 may colocalize with SMN in the cytoplasm and may be a component of the SMN complex. However, the limitation of the ICA method is an inability to resolve colocalization in components of small organelles such as the nucleus.

Keywords—Intensity correlation analysis, intensity correlation quotient.

I. INTRODUCTION

Spinal muscular atrophy (SMA) is a neurodegenerative disease and characterized by degeneration of lower motor neurons associated with muscle paralysis and atrophy, caused by deletion or mutation in the *survival motor neuron (SMN)* gene [1]. SMN is found in both the cytoplasm and nucleus of all tissues, with the greatest expression in the brain, spinal cord and muscle, and the lowest expression in the lymphocytes and fibroblasts [2], [3]. SMN is the central component of a large oligomeric complex. The SMN complex interacts with several additional proteins, including a zinc-finger protein called ZPR1, and likely functions in multiple cellular pathways, cycling between the cytoplasm and nucleus [4].

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This work is financially supported by a Research Grant for Young Scholars from Mahidol University.

In the nucleus, SMN is involved in pre-mRNA splicing. The function of SMN protein is not fully understood and further studies would help in elucidation of the tissue specificity of the disease.

Eukaryotic protein synthesis is a highly complex process and uses large quantities of cellular energy. The factors involved in aminoacylated tRNA recruitment are translation elongation factor 1A (eEF1A) and 1B (eEF1B), while translocation requires eEF2. In mammalian cells, eEF1A exists in two different isoforms: eEF1A1 and eEF1A2. The two isoforms are 92.4% identical at the amino acid level, and display equal activities in an *in vitro* translation assay [5], [6]. However, eEF1A1 is expressed ubiquitously, whereas eEF1A2 is expressed tissue-specifically in terminally differentiated cells in brain, heart and skeletal muscle [5], [7]-[9]. Although eEF1A1 and eEF1A2 proteins share similar features the function of eEF1A2 is still elusive. Loss of eEF1A2 leads to neurological abnormalities in the mice with wasted (*wst*) deletion [10]. Homozygous *wst/wst* mice appear normal until weaning age; they develop tremor and gait disturbance, followed by muscle atrophy, paralysis and death by the age of 30 days. Histological examination reveals progressive degeneration of anterior horn cells and motor nuclei in the spinal cord and brainstem, respectively [11], [12]. Neuromuscular junction (NMJ) abnormalities have also been revealed in wasted mice [12]. These data suggest that loss of eEF1A2 may be involved in the pathogenesis of motor neuron degeneration.

eEF1A is preferentially located in the cytoplasm of cells [13], [14]. Upon mitogenic stimulation, a fraction of the cytoplasmic eEF1A is shown to translocate into the nucleus in complex with the zinc finger protein ZPR1 [15]. ZPR1 is located in the cytoplasm of quiescent mammalian cells, and redistribution of ZPR1 from the cytoplasm to the nucleus can be occurred in proliferating cells by treatment with mitogens, including epidermal growth factor (EGF) [16]. ZPR1 is about 98% cytoplasmic in serum-starved cells and is about 96% nuclear in EGF-treated cells, whereas eEF1A is not detected in the nucleus of serum-starved cells and 5% of the total eEF1A is detected in the nucleus after stimulation with EGF, indicating that both ZPR1 and eEF1A are located in the nucleus of mitogen-treated cells [15]. Immunoprecipitation experiments with monoclonal antibody to ZPR1 from extracts

of [³⁵S]methionine-labeled A431 cells and western blot analysis revealed a prominent 40 kD band of SMN [9]. In addition, immunoprecipitations using anti-SMN monoclonal antibodies from [³⁵S]methionine-labeled HeLa cell cytoplasmic extracts often pull down two unidentified bands of 60 and 50 kD, which are similar to the molecular weight of ZPR1 and eEF1A, respectively [17]. These evidences raise a question whether ZPR1 and eEF1A could be part of the SMN complex.

Like ZPR1, SMN protein is present in the cytoplasm and in small subnuclear bodies, including gems and Cajal bodies [18]-[20]. One hallmark of SMA is the failure of SMN to localize within the nuclear bodies, and redistribution of SMN and ZPR1 from the cytoplasm to the nucleus is disrupted in cells from patients with SMA type I [4]. Decreased ZPR1 expression prevents SMN localization to nuclear bodies, suggesting that ZPR1 is required for SMN redistribution. It is not known whether the tissue-specific eEF1A2 isoform is in the same ZPR1-SMN pathway. Herein, a fraction of eEF1A2 redistributed to the nucleus of HeLa cells after treatment with either serum or EGF was demonstrated. Using quantitative immunocolocalization, the results also showed that distribution of eEF1A2 and SMN in the cytoplasm varies in synchrony, suggesting that a proportion of cytoplasmic eEF1A2 may colocalize with SMN.

II. MATERIALS AND METHODS

A. Cell Culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, UK) containing 4,500 mg/L D-glucose and 2 mM L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere with 5% CO₂.

B. Construction of Expression Plasmid

The strategy for construction of the plasmid pcDNA3.1(+)-C-hEEF1A2-myc was to clone full-length human eEF1A2 cDNA amplified by the polymerase chain reaction (PCR) from the I.M.A.G.E cDNA clone, pOTB7-hEEF1A2 (Clone ID 2819899; Geneservice Ltd, Cambridge, UK) into the expression vector pcDNA3.1(+)-C-myc-His (Invitrogen, Paisley, UK). The PCR primer F1 (5' -CGCGCAAGC TTAATGGGCAAGGAGAAGACCCACATC- 3') and the antisense primer R1 (5' -GGCGTTCTCGAGCGCTCTTCTTCTCCACGTTCTTG- 3') were designed to amplify the human eEF1A2 full-length cDNA from the I.M.A.G.E clone. The *Hind*III restriction site was introduced into the 5' end of the primer F1 and the *Xho*I restriction site was introduced to the 5' end of the primer R1 in order to subclone the insert into the *Hind*III and *Xho*I sites of the vector. Double strand cDNAs of 1,357 bps were synthesized by PCR at 94 °C for 2 min for a pre-heat step, followed by 94 °C for 1 min, 55 °C for 45 min, and 72 °C for 1 min for 34 cycles. The resulting PCR products

were visualized on an agarose gel and purified. The PCR products were then digested with *Hind*III and *Xho*I, and gel purified before subcloning into the *Hind*III/*Xho*I site of the pcDNA3.1(+)-C-myc-HIS vector. The expression vector was digested with *Hind*III and *Xho*I restriction enzymes, dephosphorylated with shrimp alkaline phosphatase, and then gel-purified prior to insert ligation, according to the manufacturer's instructions. The resulting clones were screened by restriction digestion with *Hind*III and *Xho*I restriction enzymes, and by colony PCR using the human eEF1A2 cDNA-specific primer F2 (5' -TCATCGGCCACGT GGACT CC- 3') and the antisense primer R2 (5' -CGATCAGCACTGCGCAGTCC- 3'). The pcDNA3.1(+)-C-hEEF1A2-myc was partially sequenced across the insert to ensure that no mutation was introduced in the PCR process of insert preparation. The T7 promoter/priming site and BGH reverse priming site located in the pcDNA3.1(+)-C-myc-His were simply chose for primer designing. The resulting sequence was aligned to the reference human eEF1A2 cDNA sequence, NM_001958, and demonstrated 100% identity.

C. Transfection

Transient transfection was performed using Lipofectamine 2000 Reagent (Invitrogen). Cells were transfected with the plasmid pcDNA3.1(+)-C-hEEF1A2-myc. The day before transfection, the cells were trypsinized, counted, and plated in 24-well plates in 0.5 ml of DMEM containing 10% FBS and without antibiotics at 8 x 10⁴ cells per well so that they were 90-95% confluent on the day of transfection. For each well of cells to be transfected, 0.8 µg of DNA was diluted into 50 µl of OPTI-MEM I Reduced Serum Medium (Invitrogen) without serum. 1.5 µl of Lipofectamine 2000 Reagent was diluted into 50 µl OPTI-MEM I Medium and incubated for 5 min at room temperature. The diluted DNA and the diluted Lipofectamine 200 Reagent were combined and incubated at room temperature for 20 min to allow DNA-Lipofectamine 2000 Reagent complexes to form. Growth medium was then removed from cells, and 0.5 ml of medium without serum was added to each well, followed by adding of 100 µl of DNA-Lipofectamine 2000 Reagent complexes directly to each well and mixing gently. The cells were incubated at 37 °C with 5% CO₂ for 4-5 h. 0.5 ml of DMEM with 20% FBS was added for a final concentration of 10% FBS. The cells were incubated at 37 °C until 24 h post-transfection or until they were ready to assay for transgene expression. For treatments with mitogens, HeLa cells grown at 37 °C in DMEM with 10% FBS were incubated in serum-free medium for 24 h, and then treated with either 10% FBS for 12 h or 100-nM EGF for 15 min at 37 °C.

D. Immunofluorescence Microscope

Cells were cultured on glass coverslips, rinsed with phosphate-buffered saline (PBS), and then fixed in cooled methanol at for 10 min at -20 °C. The cells were then permeabilized with cooled acetone for 1 min at -20 °C. The coverslips were blocked with 3% bovine serum albumin in PBS with 0.5% Tween-20 (PBS-T) for 30 min at room

temperature, and then incubated with primary antibodies for 1 hour at room temperature. Double labeling for myc-tagged eEF1A2 and SMN was carried out by sequential incubations with a monoclonal mouse anti-myc antibody (Invitrogen) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Vector Laboratories, Peterborough, UK), and then goat anti-SMN (N-19) antibody (Santa Cruz Biotechnology, Inc., Heidelberg Germany) and Texas-Red-conjugated anti-goat secondary antibody (Vector Laboratories). Antibodies were diluted in PBS to appropriate dilution: 1:5000 for anti-myc antibody; 1:500 for anti-SMN antibody; 1:500 for secondary antibodies. Cells were rinsed once and washed with PBS-T three times between each antibody incubation. The coverslips were mounted on slides using Vectashield (Vector Laboratories) with 4', 6-diamidino-2-phenylindole (DAPI), and examined by immunofluorescence microscopy using a conventional microscope Axioskop2 MOT (Carl Zeiss Inc., Thornwood, NY).

E. Quantitative Immunocolocalization

A recently developed method to test for protein colocalization by staining intensity correlation analysis (ICA) was used, and the ICA imaging was performed according to the standard method as described by Li *et al.*, using the WCIF ImageJ bundle software v1.37a (University Health Network Research, Toronto, Canada) [21]. The software is available at: <http://www.uhnresearch.ca/facilities/wcif/fdownload.html>. Briefly, cells were imaged on a Zeiss Axioskop upright fluorescence microscope. Image areas selected for analysis were converted into 8-bit monochromatic images. Fluorescence intensity was quantified in matched regions of interests for each pair of images after background subtraction and threshold adjustment. Interpretation of the ICA analysis is based on an ICA plot and an intensity correlation quotient (ICQ). The ICA plot is a scatterplot of normalized dye A versus dye B staining intensities, which provides clues to their staining relationship by representing as the product of the differences from the mean (PDM), i.e. for each pixel. The PDM value is calculated by $(\text{dye A intensity} - \text{mean dye A intensity}) \times (\text{dye B intensity} - \text{mean dye B intensity})$. The ICQ, a statistically testable quotient, is generated by subtraction 0.5 from the PDM value (to distribute the quotients to the -0.5 to +0.5 range), and used to provide an overall index of whether the staining intensities are associated in a random, a dependent or a segregated manner. With random staining $\text{ICQ} = \sim 0$; dependent staining $0 < \text{ICQ} < +0.5$, and for segregated staining $0 > \text{ICQ} > -0.5$. If myc-tagged eEF1A2 and SMN were parts of the same complex then their staining intensities should vary in synchrony, whereas if they were on different complexes of structures they would exhibit asynchronous staining, termed dependent and segregated staining, respectively.

F. Statistical Analysis

Values are present as mean + SEM. Test for significance (*p*) is with Student's *t* test.

III. RESULTS

A. Cytoplasmic Localization of eEF1A2 in control and Starved Cells

By transfecting the HeLa cells with the plasmid pcDNA3.1(+)-hEEF1A2-myc and using a specific antibody against the c-myc epitope, we showed that eEF1A2 (green), the tissue-specific isoform of eEF1A, was mainly located in the cytoplasm of the cells (Fig. 1A). In serum-starved cells, eEF1A2 was still confined in the cytoplasm (data not shown). These findings were consistent with previous studies using the mouse monoclonal antibody to eEF1A, which bind to both eEF1A1 and eEF1A2 [15].

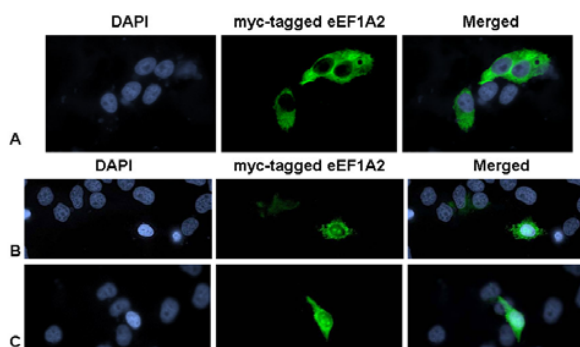


Fig. 1 Localization of eEF1A2 in HeLa cells. (A): In the presence of serum, eEF1A2 localizes in the cytoplasm of HeLa cells. In serum-starved cells, eEF1A2 is still confined in the cytoplasm (data not shown). (B-C): Redistribution of eEF1A2 from the cytoplasm to the nucleus of HeLa cell. (B): Serum-treated cells. (C): EGF-treated cells. Fluorescein (green) and DAPI (blue) represent eEF1A2 and DNA, respectively.

B. Redistribution of eEF1A2 to the Nucleus in Mitogen-Activated Cells

While eEF1A2 was not detected in the nucleus of serum-starved cells, we examined eEF1A2 localization in serum-treated and EGF-activated cells. Upon treatment with 10%FBS, a fraction of the eEF1A2 molecules was observed to redistribute from the cytoplasm to the nucleus (Fig. 1B). Diffuse cytoplasmic staining was still detected in the serum-treated cells. Similar results were obtained upon treatment of cells with EGF (Fig. 1C).

C. eEF1A2 Associates with SMN in the Cytoplasm

I previously demonstrated that myc-tagged eEF1A2 was widely distributed in the cytoplasm of HeLa cells, both in normal and serum-starved conditions. To determine colocalization of eEF1A2 with SMN, a visual inspection of the fluorescence staining pairs suggested colocalization of the proteins in the cytoplasm (Fig. 2). The ICA method was used to test a staining relationship between myc-tagged eEF1A2 and SMN. As shown in Fig. 4, the PDM plots were highly skewed toward positive values, consistent with a dependent staining pattern (Fig. 3A-E, center and right panels). Furthermore, the

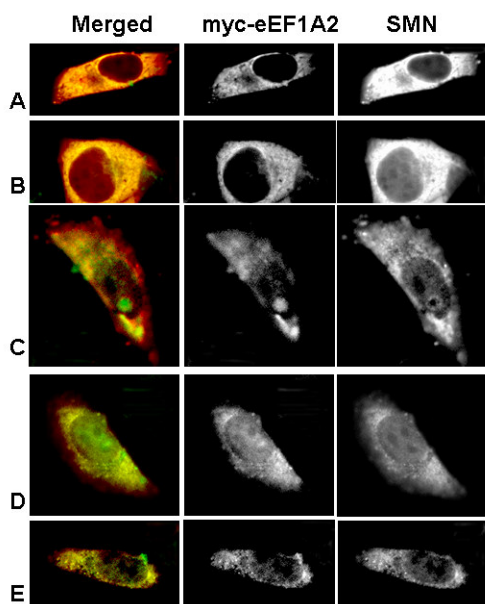


Fig. 2 pcDNA3.1(+)-hEEF1A2-myc transfected HeLa cells stained with anti-myc antibody and anti-SMN antibody. (A-E): The left panels show merged images of each analyzed cell, using the ICA method. The center and right panels show gray-scale images after background subtraction and threshold adjustment for intensity correlation analysis.

calculated ICQ values were consistently positive and significant ($+0.185 \pm 0.03$; $p = 0.0034$), indicating that the intensities in two images, green and red, varied in synchrony (i.e. they were dependent). The ICQ is based on the non-parametric sign-test analysis of the PDM values and is equal to the ratio of the number of positive PDM values to the total number of pixel values [21]. Therefore, in any image where the intensities vary together, the PDM will be positive. This analysis provided compelling evidence that staining for myc-tagged eEF1A2 varied in synchrony with that for SMN, suggesting that both proteins may colocalize in the cytoplasm.

D. eEF1A2 and SMN in the Nucleus of Mitogen-Treated HaLa Cells

In an attempt to determine whether the eEF1A2 colocalized with SMN in the nucleus of serum-treated or EGF-activated cells, the results were inconsistent (Fig. 4). In the nucleus of treated cells, a poor staining intensity relation was observed. No high pixel intensity of myc-tagged eEF1A2 was seen at both low and high SMN staining intensities. This could be a limitation of the ICQ method, e.g. perhaps an inability to resolve colocalization in components of small organelles.

IV. DISCUSSION

The eEF1A2 protein was preferentially located in the cytoplasm of cells. Herein, a fraction of eEF1A2 redistributed to the nucleus upon treatment with serum or EGF was demonstrated. This finding was consistent with previous studies that described the nuclear localization of the eEF1A

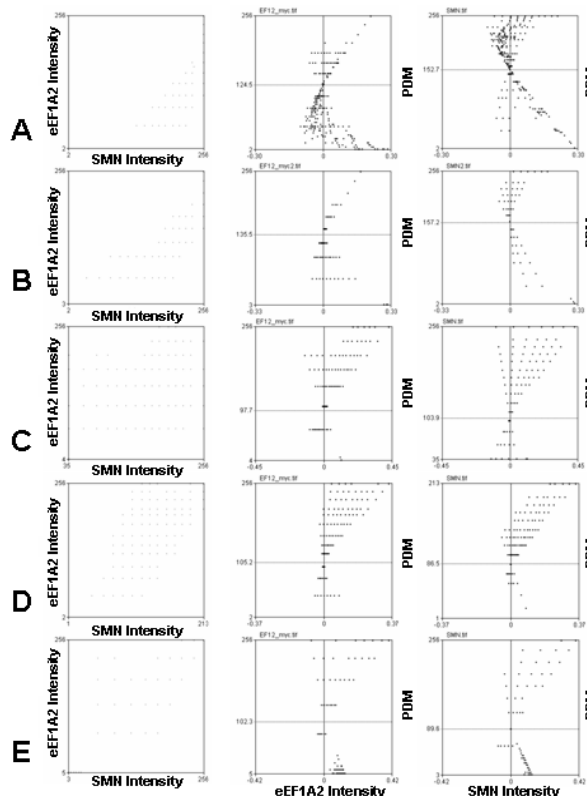


Fig. 3 Intensity correlation analysis. (A-E): Each row represents the ICA plots of the cells A-E in Fig. 2. For the center and right panels, the y-axis is the PDM (products of differences from mean) values, and the x-axis is the green (eEF1A2) or red (SMN) intensity. The left panel shows the frequency scatter plots of eEF1A2 intensity (y-axis) versus SMN intensity (x-axis). The ICQ (intensity correlation quotient) = $+0.097$, $+0.152$, $+0.183$, $+0.222$, and $+0.271$ for A, B, C, D, and E, respectively.

protein family [15], [22]. However, some studies were unable to demonstrate nuclear localization of eEF1A [23], [24]. Exp5 is shown to be a functional nuclear export receptor for eEF1A. Exp5 is also a tRNA binding protein, and this complex actively suppresses nuclear translation by promoting nuclear export of eEF1A. The reason for these opposite findings might be differences in cell culture condition. In Calado *et al.*, HeLa cells were grown in steady-state in the presence of 10% calf serum, whereas in other studies including my experiment, the cells were stressed by serum starvation prior to refeeding with serum or stimulating with mitogens [24]. It is possible that redistribution of eEF1A2 to the nuclei of stressed cells was stimulated by serum or mitogens to compensate intranuclear translation or other processes of tRNA processing. It has also been claimed recently that a significant proportion (10-15%) of cellular protein synthesis takes place in the nucleus, and occasionally reports of nuclear pools for some translation factors are proposed to support that claim [25].

Like eEF1A2, SMN is present in both the cytoplasm and the nucleus, where it accumulates in punctate structures including

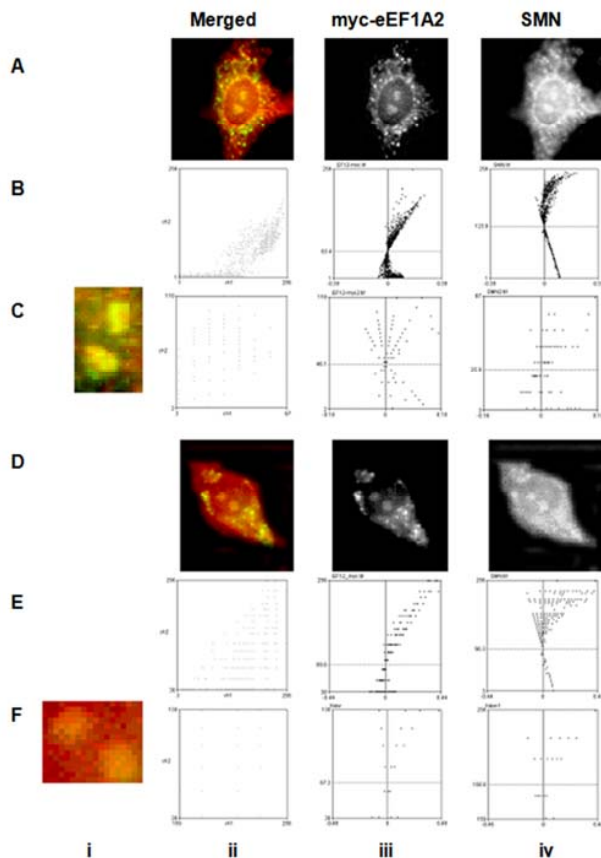


Fig. 4 Intensity correlation analysis of representative immunostaining images of two serum-treated HeLa cells stained with anti-myc antibody and anti-SMN antibody. The iii and iv panels of the row a and d show gray-scale images after background subtraction and threshold adjustment. The i panel of the row a and d shows merged images of each analysed cells. For the iii and iv panels of the row b, c, e and f, the y-axis is the PDM (products of differences from mean) values, and the x-axis is the green (eEF1A2) or red (SMN) intensity. The ii panel shows the frequency scatterplots of eEF1A-2 intensity (y-axis) versus SMN intensity (x-axis). The rows B and E represent the ICA plots of the whole cell areas. The ICQ values of the row B equal +0.271, and of E equals -0.047. The rows C and F represent images of the intranuclear areas (the i panel) and the ICA plots of the intranuclear areas (the ii-iv panels) of the cells in A and D, respectively. Note the diffuse pixel intensities in C and F.

gem and Cajal bodies [19], [20], [26]. By using double-labeling immunostaining and a quantitative analysis of staining dependency, we found that eEF1A2 and SMN exhibited a moderately strong staining intensity correlation (Fig. 4). This indicated that eEF1A2 and SMN varied in synchrony and suggested that a proportion of cytoplasmic eEF1A2 may colocalize with SMN. SMN has been demonstrated to interact with ZPR1 and the SMN/ZPR1 complex redistributes to and colocalizes to subnuclear structures [4]. It has been postulated that upon EGF stimulation, ZPR1 binds to eEF1A and SMN in the cytoplasm and redistributes to the nucleus [27]. Immunoprecipitations with anti-SMN antibodies have been shown to pull down two unidentified bands with mobilities

very similar to those of ZPR1 and eEF1A [17]. Based on quantitative immunocolocalization as described above, it is possible that eEF1A2 could be part of the so-called SMN complex.

It has previously been shown that NMJs in wasted mice shows striking abnormalities of terminal axons accumulated with neurofilaments (NF), with reduction of branched structures of the subneural apparatus [12]. Moreover, no axonal sprouting is detectable. These abnormalities are similar to those found in *Smn* mutant mice, but contrast with *SOD^{G93A}* transgenic mice, indicating that NMJ changes in wasted and *Smn* mutant mice may be caused by a common axonal degenerative process [28]. As cytoskeletal components play a major role in axonal growth and plasticity, abnormal accumulation of NF in terminal axons of wasted and *Smn* mutant mice may contribute to the defect in terminal axonal growth and plasticity. The resulting aberrant cytoskeletal organization at the NMJ of wasted and *Smn* mutant mice may contribute to the loss of motor neuron function.

The association of eEF1A2 with SMN in the nucleus of serum-treated cells was next examined. Unfortunately, there were no satisfactory ICA results to determine their relation. This is possible that a fraction of cytoplasmic eEF1A2 that is translocated into the nucleus is small, and only a tiny proportion of this fraction may colocalize with SMN, or that interaction between eEF1A2 and SMN in the nucleus exists only in a particular stage of the cell cycle.

The ICA-ICQ costaining analysis is a key element of this study. It was used for the first time to study colocalization of syntaxin 1, G_{α_o} , and N-type calcium channel complex at a tissue level, a presynaptic nerve terminal [21]. This technique is distinguished from the usual dye-overlap method by its focus on the variations in protein concentrations across the cell and not simply their locations. Perhaps the worst limitation of the dye-overlap method is that when one protein target is distributed across most, or all, of the cell, a second protein will be identified as colocalized even when its distribution is totally random with respect to the first. As described by Li *et al*, the ICA method avoids this mistake because if a randomly distributed protein overlaps with a broadly staining one, it will not be reported as associated because it does not vary in synchrony [21]. The ICQ in this method serves as a statistically testable, single-value assessment of the relationship between the stained protein pairs. ICA-ICQ analysis has advantages over the conventional immunoprecipitation method in identifying potential low-affinity protein complexes and in retaining information on the cellular and subcellular location. Its limitation is an inability to resolve colocalization of two proteins that are components of a small organelle, which might be a cause of the poor staining intensity relation that is found when analyzing colocalization of myc-tagged eEF1A2 and SMN in the nucleus.

Implications of these observations remain to be explored. Further studies aimed at the confirmation of the protein-protein interaction using the yeast two-hybrid technique and

the identification of the role played by interaction between SMN and eEF1A2 would not only represent a major advance in our knowledge of the pathophysiology of SMA, but could also provide the basis of the therapeutic approach for this disease.

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