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Detection of newly synthesized proteins in cancer cells: A practical approach

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Abstract: Up to 30% of newly synthesized proteins in cells are DRiPs/RDPs (Ribosomal Defective Products/Rapidly Degraded Proteins). They are a special subset of nascent proteins that never attain their native structure and are rapidly degraded after biosynthesis. The major degradation pathway of DRiPs/RDPs involves classical ubiquitin-26S proteasome pathway but their smaller fraction is eliminated by 20S proteasome without ubiquitin tagging.
The increasing interest on DRiPs/RDPs biological implications and their kinetic nature results in invention of several detection methods. Here we provide characterizations and comparison between three most common pulse-chase radiolabeling icnl. radioisotopes containing amino acids 35S-Methionine 3H-Leucine as well as novel non-radioactive technique using biorthogonal amino acid tagging and click-IT chemistry. Moreover, we discuss cyto-physiological properties of DRiPs/RDPs as the main source of MHC class I antigens and contribution of newly synthesized proteins to anticancer action of proteasome inhibitors.
Finally, in practical approach we show that azidohomoalanine (AHA) metabolic labeling of murine colon carcinoma cells C26 for 5h followed by ethanol-based fixation and Click-IT detection by Alexa Fluor 555 alkyne methods can be used for *in situ* histochemical visualization of synthesized proteins by means of Laser Scanning Confocal Microscopy. We visualized the forming aggresome in C26 cells upon proteasome inhibition. This aggresome was composed of the inner dense core and the mantel of labeled proteins. Thus this method can be a valuable tool in anticancer research on proteasome inhibitors.

1. Introduction

Proteins are the main building blocks forming the structure of all living organisms. They are composed of amino acids and are the products of complicated intracellular machinery, in which DNA code is transcripted into mRNA and subsequently translated into polypeptide chain consisted of covalently linked amino acids. This step takes place on ribosome in ATP-dependent manner. The nascent protein must undergo proper folding to obtain unique three-dimensional conformation with the additional help of molecular chaperons [1]. This multistep process of protein production makes it susceptible to errors. Any mistake in synthesis may result in misfolding and loss of macromolecule function. The errors potentially generated are numerous: mutations, translation errors, age related errors, exposure to environmental stress conditions such as heat, metal ions, oxidation are the most prevalent [2]. Therefore, cells adopted various quality control systems to prevent generation and accumulation of harmful misfolded proteins in cells [3]. Two general systems acts parallel in cellular proteolysis: lysosomal-based degradation incl. authophagy and Ubiquitin-Proteasome System (UPS) [4]. The majority of misfolded proteins undergoes UPS- degradation [5].

The special subset of proteins was distinguished in nascent proteins: Defective Ribosomal Products (DRiPs). DRiPs are defined as proteins that never attain their native structure and are rapidly degraded after biosynthesis [6, 7]. The similar term Rapidly Degraded Polypeptides (RDP) are also used in regard to this pool of short-lived proteins [6, 7].

The aim of this article is to discuss and compare the published methods of nascent protein detection pointing a special attention to their degradation pathways. Moreover we demonstrate a practical approach of newly synthesized protein visualization during aggresome formation in C26 cancer cells by Laser Scanning Confocal Microscopy.

2. Methods

2.1. Search strategy and selection criteria

Our research strategy was aimed at evaluating studies for detection and visualization of nascent protein degradation. The terms *“nascent proteins”, ”newly synthesized proteins", "Short-lived proteins" "Defective Ribosomal Products (DRiPs)",* as well as *"protein degradation", "proteasome", "Ubiquitin-Proteasome System (UPS)”,* were searched in PubMed (NCBI) and Google Scholar. Scientific articles from 1971 to 2018 were searched.

3. Experimental Materials and Methods

3.1. Reagents

L-azidohomoalanine (Click-iT® AHA C10102 ThermoFisher) was dissolved with DMSO to 50mM stock solution. Alexa Fluor® 555 alkyne (A20013 ThermoFisher) was dissolved in Wather/gicerol (1:1 solution) to the recommended final concentration of 2.5mM. The proteasome inhibitor MG-132 (Calbiochem/ Merck) was dissolved on DMSO to the final concentration of 10mM. All reagents were Aliquoted and stored at –20°C until use.

3.2. Cell culture

Murine adenocarcinoma C26 cell line was obtained from ATCC. Cells were grown in RPMI1640 (Biochrom, Germany) with stable glutamine and supplemented with 10% FCS and standard antibiotics. Cells were cultured in RPMI-1410 medium with stable glutamine (Biochrom, Germany) supplemented with 10% heat-inactivated FCS (Biochrom, Germany), 1% Antibiotic–Antimycotic (Thermo Fisher/Life Technologies, US) in 25 cm^2 tissue flasks (Greiner, Germany) and kept at 37°C in a 5% CO_2 humidified incubator and passaged every three days with EDTA-Tripsin solution (Biochrom).

4. Metabolic labeling and cytochmistry

C26 cells grown onto multi-chamber slides (Becton Dickinson) and before metabolic labeling were staved for 30 min in RPMI-1410 Methionin-free medium (Sigma). The medium was replaced 2 times to eliminate the residual methionin before adding L-azidohomoalanine. After starvation L-azidohomoalanine was added to the final concentration of 0.25mM.

Cells were incubated at 37°C, 5% CO_2 with L-azidohomoalanine (AHA) for 5h in the presence of proteasome inhibitor MG-132 in the concentration of 10mM to prevent degradation of newly synthesized proteins. After metabolic labeling were fixed using the modified routine procedure for cytopathology with ice-cold buffered 70% ethanol (pH 7.5; phosphate buffer) for 3 minutes and directly rehydrated (pH 7.5; phosphate buffer). Washed once with 3% BSA in PBS. Subsequently, 1 µM Alexa Fluor® 555 alkyne in Click-iT® cell reaction cocktail with Copper (II) sulfate (C10269 ThermoFisher) was added or 30 minutes at room temperature in darkness. Cells were washd three times with 1BSA in PBS and embedded in Vecta Shield containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, US).

The analysis and images were made using a confocal laser SP5 microscope equipped with appropriate lasers (HeNe 633nm, DPSS diode 561nm, and Argon laser: line 488nm) and Las-AF software (Leica, Germany).

5. The state of Art

5.1. The Ubiquitin-Proteasome system in degradation of nascent proteins

UPS is an conservative proteolytic system for protein clearance in eukaryotic cells. This is composed of ubiquitin conjugation machinery and the proteasome as an proteolytic complex degrading the targeted protein into oligopeptides. Ubiquitin is a 76-amino-acid polypeptide, which is covalently attached to the protein in a form of a polyubiquitin chain serving as a degradation signal. The polyubiquitination is achieved by cascade of enzymes: the ubiquitin-activating

enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s) The ubiquitin is activated by E1 enzyme than transferred onto E2. Finally E3 enzyme catalyzes the conjugation of ubiquitin to the target protein. The activation of ubiquitin catalyzed by the E1 enzyme is ATP dependent and leads to formation of high-energy thioester bond [8].

The ubiquitin is attached to the lysine of the targeted protein but there has been found that also serine, threonine and cysteine may be modified by ubiquitin [9]. Within ubiquitin polypeptide there are 7 internal own lysine residues, each of which can be used for bonding another ubiquitin molecule and create polymeric ubiquitin chain. Thus there can be formed various poly ubiquitin chains but the chain linked by lysine 48 (K48 chains) predominantly serves as a signal for proteasomal degradation [10, 11, 12].

The 26S proteasome is a multisubunit multicatalytic complex composed of 20S catalytic core and two 19S regulatory particles forming the 26S proteasome complex [13]. The 20Sproteasome is a central cylinder formed from multiple subunits arranged into four heptameric rings with three main proteolytic activities: trypsin-like, chymotrypsin-like and caspase-like attributed to the β1, β2 and β5 subunits respectively [14]. The 20S core proteasome displays itself the ability to degrade unfolded polypeptide chains without prior polyubiquitination [15]. The 19S cup complexes contains six-subunit protein ring, which occupy each end of the cylinder. The cup structure is a link between arriving ubiquitinated protein and central proteolytic core. It regulates opening of a gate to 20S proteasome complex and unfold proteins in ATP-dependent manner. Each of 19S particles contains ubiquitin receptors, which can bind approaching proteins and a metalloprotease removing polyubiquitin chain before protein degradation [16, 17]. In the case of defective protein that cannot be successfully folded by molecular chaperons the CHIP co-chaperone performs ubiquitinylation of targeted protein that is transferred to 26S proteasome for degradation [18]. However severe misfolded proteins can be degraded by 20S Proteasome co-translationally [7].

Inhibition of the proteasome leads to accumulation of newly synthesized proteins and UPS components in the aggregates, which can be observed within the cell as single round structures termed aggresomes, which initially assembles in the peri-centriolar regions of the cell [19, 20, 21].

5.2. Other extralysosomal proteolytic systems

The function of UPS is supplemented by several proteases. TPPII is the biggest known peptidase forming self-compartmentalizing, twisted double stranded cytosolic peptidase complex of 6MDa [22]. It displays both exo- and endo-proteolytic activities. Thus it provides support and even substitutes some of the proteasomal functions. It can supplement the downstream action of the proteasome in sequential protein degradation through trimming of tripeptides from oligopeptides released by the proteasome [23], [24]. The products released by 20/26S proteasomes are oligopeptides comprised of 4 to 25 AA [13, 14]. In order for complete degradation TPPII digests substrates longer than 15 AA [25, 26]. TPPII was described as being involved in several processes of cell regulation [22, 27]. Recently, TPPII was shown to be recruited into aggresomes in colon cancer C26 cells upon proteasome inhibition [28] and semispecific TPPII inhibitor induces protein aggregation in leukemic U937 cells [29].

The UPS cooperates also with an ER-associated degradation (ERAD) in degradation of defective proteins form endoplasmic reticulum. There are 3 types of this pathway: ERAD-L for misfolded luminal Endoplasmic Reticulum proteins, ERAD-M for ER proteins with error in membrane domain and ERAD-C for ER proteins with unfunctional cytosolic domain ERAD systems contain enzymes machinery, which is able to recognize misfolding, prevent leaving invalid proteins from ER to excretory pathway, export it back to the cytosol by retrotranslocation and lead to degradation [2].

5.3. The origin of newly synthesized proteins and their degradation pathways

From 70th XX century on the basis of degradation kinetics cellular proteins are divided into two main groups of proteins: “short-lived” (with a fast degradation rate) and “long-lived” (with a much slower degradation rate) [30, 31], [32]. Both kinetics of degradation were separated by a clear discontinuity and these two groups of proteins have been identified in all cells.

The studies revealed that 30% of newly synthesized proteins are eliminated with a half-life <10 minutes and this part of extremely short-lived proteins was named DRiPs (defective ribosomal products)/RDPs (rapidly degraded proteins) [6, 7]. 75% of these proteins are eliminated by 26S proteasomes and remaining 25% by 20S proteasome complex independently of 19S regulators [7]. DRiPs/RDPs can be divided into two classes based on their solubility. The soluble RDPs fraction is degraded by ubiquitin-depended manner, which is linked with 26S proteasome but insoluble fraction seems to be degraded in a different way without involvement of ubiquitin. Qian et al. bring proofs and conclude that 75% of DRiPs/RDPs are degraded by 26S proteasome Hsc70-depended pathway based on ubiquitination. The remaining 25% are degraded without ubiquitin-link by 20S proteasome without involvement of 19S regulatory subunit and are largely unaffected by Hsc70 modulatory activity [7]. DRiPs/RDPs are also the largest fraction of proteins degraded by proteasomes and constitute 70-75% of all the protease complex substrates [33, 34].

Figure 1. DRiPs/RDP and their degradation pathways
DRIPs/RDPs are degraded co-translationally. The larger fraction of them one is water soluble and undergoes polyubiquitination prior to degradation by 26S proteasome. The minor fraction (ca 25%) is digested directly within 20S core of proteasome without previous ubiquitinylation. Most generated oligopeptides is a source of MHC class I epitopes (vial or cancer antigens).
Original Figure.

5.4. DRIPs and immunity

At the beginning DRiPs were defined as ribosomal products that never attain their mature structure because of defective mRNAs [35, 36], ribosomal frame shifting [37, 38], tRNA-amino acid misacylation [39], transcription errors [40] or all other mistakes during converting DNA information into protein [41]. This definition implicates that RDPs/DRiPs are accidental products of protein synthesis but recent studies have proven that they're not only a junk biosynthesis consequence but also have a crucial contribution to cellular processes. Thus, DRiPs/RDPs was considered only as abnormal, misfolded proteins but now it is known that they are a main source of cellular and viral antigens

presented by histocompatibility complex class I molecules: viral and cancer [6, 42].

Yewdell concluded form Dolans studies [33, 43] that even 70% of MHC class I complexes can originate from DRiPs. This remark is also supported by interesting observation of immunologic response for virus-infected cells. CD8+ cytotoxic T-lymphocytes recognize specific antigens on the surface of infected cell simultaneously to virus is entrance and long before source protein could be detected within infected cell. Transition of viral protein into antigen should take much longer and probably is not responsible for activation of early cytotoxic T-lymphocytes. However antigens for MHC class I presentation can be also derived from long lived proteins but DRiPs generation seems to be favored for rapid recognition of infected cells by CD8+ T cells [41]. DRiPs were also described as a major contributor to the antigenic presentation associated with immunological tolerance [44]. Noticeable, the role of rapidly degraded proteins indicates possibility for preconcerted RDP-biosynthesis, which is not only an error effect. Referring to this hypothesis, scientists have indeed found unique subset of ribosomes specialized for antigenic relevant DRiPs synthesis named immunoribosomes [45, 46]. Proteins produced by those complexes are believed to be rapidly degraded by (immuno)proteasomes into oligopeptides dedicated to antigen generation. There exists hypothesis for close relationship of both of them [47], which is supported by discovery of several proteins capable to interact with the both complexes [48, 49] for example eIF1A [50].

5.5. Newly synthesized proteins and Cancer

Current established therapeutic procedures include inhibition of the proteasome as anticancer strategy [51]. Inhibition of proteasome proteolysis leads to misfolded protein response and accumulation of newly synthesized proteins that induces apoptosis. Thus most sensitive to proteasome inhibition are cells that perform intensive protein synthesis like plasmmocytoma cells.

Upon proteasome inhibition newly synthesized ubiquitinated proteins are transported along microtubules to the pericentriolar proteolytic center of the cell and stored there as an aggresome [19-21]. The accumulation of misfolded proteins within the aggresome is one of the postulated mechanisms of action of proteasome inhibitors as anticancer drugs. Nonetheless, aggresome disruption by histone deacetylase inhibitors [52] and microtubule acting agents [53] can augment the anticancer activity of proteasome inhibitors. Thus, aggre-some formation in cancer cells likely protects them from proteasome inhibitor-induced unfolded protein stress, and therefore can limit anticancer effects of proteasome inhibitors.

5.6. Comparison of DRIP detection protocols

The biological features and function of DRiPs/RDPs becomes a substantial subject of studies recently because of their serious impact on cell biology. As mentioned before, DRiPs/RDPs are nascent proteins with a half-life < 10mins a and can constitute up to 30% of newly synthesized proteins [6, 7]. Thus due to extremely short live time of DRiPs/RDPs, their experimental detection is difficult, and several studies were focused on acquiring the best way of their detection. Various protocols were used to achieve this aim.

The most common method is classic radioactive pulse-chase analysis [7]. The method involves labeling of newly synthesized proteins with radioactive amino acids which allows quantitative analysis of the fate of given proteins in the time-depended manner [54]. The detection of radioactive protein can be subsequently preformed by either liquid scintillation counter or autoradiography. Alternatively non-radioactive amino acids surrogates such as L-azidohomoalanine (AHA) can be used for metabolic labeling of proteins followed by detetection by selective click-chemistry techniques.

Pulse-chase radiolabeling of newly synthesized proteins is a well-known method for DRiPs/RDPs analysis. First step of the method pulsing of cells for a short time period with special medium that contains radiolabeled amino acids. The most common radioactive amino acid used in this method is 35SMethionine. It is used due to its high specific activity (>800Ci/mmol) and quick detection. However, potential disadvantage of this amino acids is its low abundance in proteins (~1.8% of the average amino acid composition) [55] thus high radioacivity must be used up to 5 mCi/ml [6]. Alternatively

3HLeucine can be used for radioactive protein labeling. Because of DRiP high turnover rate pulse-labeling time has to be short enough for appropriate detection. Qian et al. measured nascent proteins degradation by radiolabeling in HeLa cells with 35s Met for 5 min [7]. Furthermore, Shubert with colleagues did it for only 30 seconds in their studies [6].

The second step of the technique contains chasing of radioactive palsed cells in complete medium with unlabeled methionine for longer period of time to allow degradation of newly synthesized proteins. Qian et al. chased cells for up to 4h and Shubert et al. did it for up to 60 min.

At the end the detection of signal can be made by measuring radioactivity present in trichloroacetic acid precipitates of whole cell lysates and culture media [7]. Measurement can be done by scintillation counter or alternatively by exposing dried gel from SDS-PAGE to phosphor imager screen or X-ray film [56].

Alternatively 3HLeucine radioactive protein labeling was used by Knecht's group for pulse-chase analysis of newly synthesized [57]. The degradation of short-labeled proteins was examined in fibroblasts by release of trichloroacetic acid-soluble radioactivity. Fuertes at al. estimated DRiP rate to about 30% of newly synthesized. However half-life observed longer up to 1 h probably due to longer tilme of the radioacive pulse. They also calculated that proteasomes are responsible for degradation go 60% newly synthesized proteins. The described methodology has a high accuracy but working with radioactive materials is potentially dangerous for experimenter and environment especially with long-lasting 3H nuclide.

Details and comparison of the mentioned protocols is displayed in Table 1.

Table 1. Comparison of most common DRiPs/RDPs detection protocols

	Shubert et al.	Qian et al.	Fuertes et al.
Cell line	HeLa	HeLa	Human fibroblasts
Pulse time Chase time	30s 60min	5min 4h	15min Different chase time up to 25h
Source of radioactivity	[35S]methionine 5 mCi/ml	[35S]methionine	[3H]leucine or [3H]valine 1 mCi/ml
Detection	SDS-PAGE and radioactivity measurement in dried gel	TCA precipitation and scintillation counting/ SDS-PAGE and radioactivity measurement in dried gel	TCA precipitation and scintillation counting
T1/2	10min	7.6min	1.1h

5.7. Click-IT chemistry in detection of newly synthesized proteins

Recently new method for detecting on synthesized proteins was described. It allows to detect proteins of interests without using radioisotopes. Thus, it is safer for environment, experimenters and furthermore enables *in situ* histochemical visualization. Bioorthogonal noncanonical amino acid tagging – BONCAT is a novel method based on application of amino acid surrogates modified with azide or alkyne groups, which are incorporated into proteins during biosynthesis [58]. It is possible because certain tRNA synthetases employed to the process are unable to recognize natural amino acids and can recognize those with slightly different structure [59]. Bioorthogonal molecules are characterized as synthetic, not biologically synthesized and not interfering with native biochemical processes. They are frequently analogs of native biomolecules with addition of special groups required for click chemistry [60]. There have been described a series of biorthogonal amino acids which are able to successfully compete with native amino acid molecules during translation but there are only several molecules, which can be used by translational machinery without genetic modifications of the host cell [61]. One of the most efficient, often described as a member of this subset is an amino acid substitute L-azidohomoalanine (AHA) – an analog of L-methionine [62]. An azide group of AHA incorporated into proteins makes them distinct from other polypeptides in cell and enables selective click chemistry-mediated detection [60]. The functional azide group reacts with alkyne through 1,3-dipolar cycloaddition to yield triazole linkage. The reaction is catalyzed by Cu(II) and here we came across the serious disadvantage – copper catalyst in higher concentration is toxic to cells. To circumvent the problem several metal-free azyde-alkyne

cycloadditions have been developed [63]. The whole process allows to visualize proteins of interest by many methods e.g. microscope in vivo, SDS-PAGE due to azide-alkyne fluorescence dye linkage. The 1,2,3-triazole linkage is extremely strong and resistant to hydrolysis, oxidation, reduction or ionization in mass spectrometry analysis. Finally, proteins labeled with AHA can be detected using subsequent analysis by flow cytometry, imaging or standard biochemistry techniques such as gel electrophoresis. Detection sensitivity obtained in this process is similar to that in radioactive 35S Met method and is compatible with downstream LC-MS/MS and MALDI MS analysis [64].

6. The Practical Approach of newly synthesized protein visualization in cells

We show localization of newly synthesized proteins using amino acid analogue AHA visualized by Click chemistry with fluorochrome within murine colon adenocarcinoma C26 cells.

C26 cells were chosen for this practical approach due to their properties. In our laboratory we investigated anticancer properties of various proteasome inhibitors against many cancer cell lines and among them C26 cells respond to proteasome inhibition by formation of the giant aggresomes and can survive long-term proteasome inhibition making them an ideal candidate for protein aggregation study. The aggregate is formed in perinuclear region and fully mature easy to detect large and round is formed within 12-24 hours of proteasome inhibition (unpublished labor data).

7. Results

As it is shown in Fig 1 after 5h of proteasome inhibition by 10μM MG-132 synthesized proteins (red) accumulate in the cytoplasm of C26 cells. They predominately accumulate in a one single round structure in perinuclear region corresponding to forming aggresome (white arrows in panel B). The forming aggregate is composed of a dense solid core (arrows in panel C) with a mantle containing less dense material (arrowheads in Panel C). Moreover, the residual part of cytoplasm is stained less intensively. There was no signal within nuclei observed.

8. Short Discussion and Conclusion

Non-radioactive by Click-IT a chemistry-based method of newly synthesized protein detection gives an interesting alternative for their radioactive detecting. Moreover Click-IT methods have an additional advantage because they can be used in situ histochemical visualization of synthesized proteins. Moreover, our validation endorsed the ethanol based fixation suitable for this application. We visualized the forming aggresome in C26 cells upon proteasome inhibition. This aggresome was composed of the inner dense core and the mantel of labeled proteins.

Concluding this method can be a valuable tool in anticancer research on proteasome inhibitors incl. aggresome formation studies.

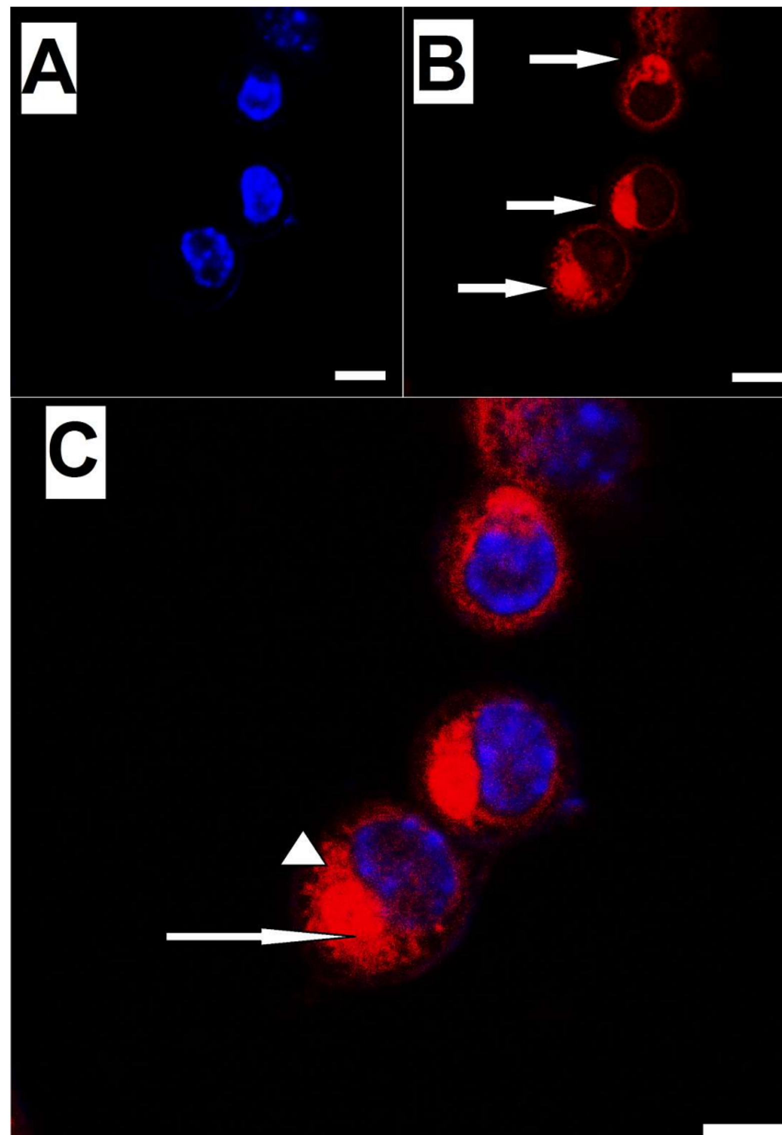

Figure 2. Visualization of newly synthesized protein in C26 mouse adenocarcinoma
C26 cells were treated for 6h with 10uM Mg132 to inhibit protein degradation. Synthesized proteins were detected by AHA incorporation (for 6h) and visualized by Alexa Fluor 555 alkyne after ethanol-based fixation using confocal microscopy. A. Nuclei - Blue - nuclei stained with DAPI; ;. Synthesized proteins - Red – proteins with incorporated AHA; C. Merge. Scale bar: 7.5μM.

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