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Daphne Nikles, Karen Vana, Sabine Gauczynski, Heike Knetsch, Heike Ludewigs, et al.. Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2008, 1782 (5), pp.335. 10.1016/j.bbadis.2008.02.003 . hal-00501570

HAL Id: hal-00501570

<https://hal.science/hal-00501570v1>

Submitted on 12 Jul 2010

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Accepted Manuscript

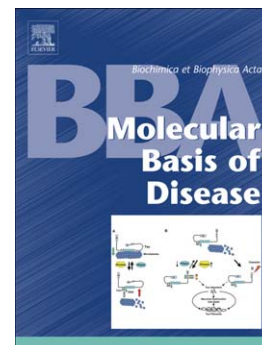
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PII: S0925-4439(08)00054-9
DOI: doi: [10.1016/j.bbadis.2008.02.003](https://doi.org/10.1016/j.bbadis.2008.02.003)
Reference: BBADIS 62793

To appear in: *BBA - Molecular Basis of Disease*

Received date: 23 January 2008
Revised date: 12 February 2008
Accepted date: 12 February 2008



Please cite this article as: Daphne Nikles, Karen Vana, Sabine Gauczynski, Heike Knetsch, Heike Ludewigs, Stefan Weiss, Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins, *BBA - Molecular Basis of Disease* (2008), doi: [10.1016/j.bbadis.2008.02.003](https://doi.org/10.1016/j.bbadis.2008.02.003)

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Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins

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Running head: Subcellular localization of the 37kDa/ 67 kDa LRP/LR and PrP

Keywords: prion protein; PrP; laminin receptor; LRP/LR; subcellular localization; cancer; heterochromatin

Abbreviations: LRP/LR, laminin receptor precursor/laminin receptor; EGFP, enhanced green fluorescent protein; ECFP, enhanced cyan fluorescent protein; PrP, prion protein; PrP^c, cellular form of PrP; *Prnp*, PrP gene; Δ SP-PrP mutant, PrP mutant lacking its signal peptide; kDa, kilodalton; siRNA, small interfering RNA

Abstract

The 37 kDa/67 kDa laminin receptor LRP/LR acts as a receptor for both PrP^c and PrP^{Sc} at the cell surface. Here, we further analyzed the subcellular localization of fluorescent labeled prion protein (PrP) and laminin receptor (LRP/LR) molecules. We show that EGFP-PrP is localized at the cell surface and in a perinuclear compartment, respectively. In contrast, a DsRed- Δ SP-PrP mutant lacking the signal peptide is almost exclusively found in the nucleus but does not colocalize with heterochromatin. Interestingly, LRP-DsRed efficiently colocalizes with EGFP-PrP in the perinuclear compartment and LRP-ECFP partly colocalizes with DsRed- Δ SP-PrP in the nucleus, respectively. We conclude that the interactions of PrP and LRP/LR are not restricted to the cell surface but occur also in intracellular compartments suggesting a putative role of LRP/LR in the trafficking of PrP molecules.

1. Introduction

The cellular prion protein PrP^c is one of the most studied proteins in eukaryotic cell biology, because it is not only the central player in prion diseases but it unraveled how an infectious agent could consist of protein only. The so-called protein-only hypothesis, which was formulated by Stanley B. Prusiner in 1982 [1] is nowadays well-grounded on recent studies [2, 3]. However, it still remains an open task to determine the function of PrP^c, because *Prnp* knock-out mice showed no obvious phenotype [4]. There exist a tremendous number of postulated functions for PrP^c [5-11] including copper binding, signal transduction, neuroprotection and oxidative stress reactions.

Previously, a green fluorescent protein coupled PrP^c has been used in many studies for subcellular visualization of the protein [12-16]. In living cells, immunofluorescent studies showed an equal localization pattern compared to endogenous PrP [17]. Interestingly, a mutant version of PrP^c lacking its signal peptide translocates the protein from the endoplasmatic reticulum to the nucleus [18]. However, the finding cannot be explained by the presence of nuclear localization signals within the protein sequence [18]. Since PrP^c has RNA binding properties [19], the nuclear localization of truncated PrP^c may be of physiological relevance.

The 37 kDa/67 kDa laminin receptor (LRP/LR) plays a major role within the cellular functions of PrP^c. The protein is a multifunctional type II transmembrane protein with roles in ribosomal transcription, cell attachment, tumor metastasis formation and functions as a receptor for various viruses (for review: [20]). LRP/LR acts as a cell surface receptor for PrP^c [21] and for infectious prions [22, 23]. Recently, several approaches for prion disease therapy targeting LRP/LR have been described (for review: [20, 24, 25]). These include (i) antibodies directed against the 37 kDa/67 kDa LRP/LR [26, 27], (ii) siRNAs targeting the 37 kDa LRP mRNA [28] (Ludewigs et al., in preparation), (iii) transdominant negative LRP/LR mutants acting as a decoy [29] and (iv) polysulfated glycanes [22]. Blocking or downregulation of LRP/LR by these molecular tools reduced invasion of tumorigenic fibrosarcoma cells (HT1080) recommending these molecules as alternative therapeutics for prevention of cancer metastasis and tumor progression [30]. In the present study, we used both fluorescence-coupled PrP^c and LRP/LR to visualize both proteins after transfection in eukaryotic cells. We generated cyan fluorescent LRP (LRP-ECFP), red fluorescent LRP (LRP-DsRed) and green fluorescent LRP (LRP-EGFP), respectively, as well as a red fluorescent prion protein mutant lacking its signal peptide (DsRed- Δ SP-PrP). In addition, we used full-length prion protein

coupled to the green fluorescent protein (PrP-EGFP) [17]. Here, we describe the subcellular localization of these proteins after transient transfection in two different cell lines employing confocal immunofluorescence (IF) microscopy. We confirm that PrP-EGFP is localized in a perinuclear compartment and on the cell surface. Moreover, we show that DsRed- Δ SP-PrP is translocated into the nucleus without colocalizing with heterochromatin. Additionally, we observe a colocalization of LRP-DsRed and EGFP-PrP in the perinuclear compartment and a partly colocalization of LRP-EGFP and DsRed- Δ SP-PrP in the nucleus suggesting a potential role of PrP^c in gene regulation.

2. Material and Methods

2.1. Plasmid Constructions

For the generation of the plasmid pDsRed- Δ SP-PrP, the coding sequence for DsRed was amplified from the plasmid pDsRed-Express-N1 (Clontech) using the oligonucleotides 5'-AgeI-DsRed (5'-GTCAAACCGGTCGCCACCATGGC-3') and 3'-BgIII-DsRed (5'-GTCAAAGATCTCA GGAACAGGTGGTGGCG-3') and subsequently inserted into the target vector pEGFP-PrP [17] using the restriction sites *AgeI* and *BgIII*. The plasmid pLRP-DsRed was cloned by inserting the murine laminin receptor coding sequence (amplified from pSFV1-moLRP::FLAG [22]) into the target vector pDsRed-Express-N1 (Clontech) by use of *BamHI*- and *SmaI*-coding oligonucleotides 5'-*BamHI*-LRP and 3'-*SmaI*-LRP (5'-GTCAAGGATCCGCCACCCATGTCC-3', 5'-GTCAACCCGGGGCCACCATGTCCGGAGCC-3'). The plasmid pLRP-EGFP was generated by insertion of the LRP cassette amplified from pSFV1-moLRP::FLAG using the oligonucleotides 5'-*XmaI*-LRP (5'-GTCAACCCGGGGCCACCATGTCCGGAGCC-3') and 3'-*BamHI*-moLRP (5'-GTCAAGGGATCCGGACCACTCAGTGGTGGC-3') into the plasmid pEGFP-N3. The vector pLRP-EGFP was cloned in two steps. First, pEGFP-N1 plasmid was generated via exchange of DsRed with the ECFP cassette amplified from pEGFP-Endo vector (Clontech) with the primers 5'-AgeI-EGFP (5'-GTCAAACCGGTCGCCACCATGGTGG-3') and 3'-NotI-EGFP (5'-GTCAAGCGGCCGCTACTTGTACAGCTCGTCCATG-3') and subsequent insertion into pDsRed-Express-N1 vector. In the second step, the murine LRP sequence was inserted into the newly generated vector pEGFP-N1 by restriction of the pLRP-DsRed plasmid using the restriction enzymes *XmaI* and *AgeI*. All generated plasmid sequences were verified by dideoxy sequencing.

2.2. Cell Culture and Transfections

Baby hamster kidney (BHK, ATCC CCL-10) and murine neuroblastoma cells (N2a, ATCC CCL-131) were cultivated in DMEM (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and non-essential amino acids. For calcium phosphate transfection, cells were plated at 30% confluency in 6 well plates prepared to contain one coverslip per well and were incubated overnight to enable cell attachment. In total, 8 μg of mixed plasmids plus 9 μl (2.5 M) calcium chloride completed to 90 μl with ultrapure water was prepared. Next, 90 μl of sterile filtered HBS buffer (sodium chloride 281 mM, HEPES 100 mM, sodium phosphate 1.5 mM, pH 7.12) were added while plasmids were vortexed for one minute and precipitates were added onto the cells containing 25 μM chloroquin in DMEM. Media was changed once after 8 hours and a second time after overnight incubation. Transfection experiments were carried out in three individual experiments.

2.3. Western Blot Analyses

48 hours after transfection, cells were detached by trypsin (0.1% in PBS) lysed in 50 μl lysis buffer (10mM Tris/HCl pH 7.5, 100mM NaCl, 10mM EDTA, 0,5% Nonidet P-40, 0,5% Desoxycholate) and incubated on ice for 30 minutes. Samples were centrifuged at 14.000 rpm in an Eppifuge (Heraeus) and supernatant was mixed with SDS sample buffer. Samples were boiled at 95 $^{\circ}\text{C}$ for 5 minutes and subjected to SDS polyacrylamid gel electrophoresis using a 12% separation gel. Samples were blotted on nitrocellulose membranes and blocked by horse serum (20% in PBS) followed by incubation with either anti-PrP antibody SAF 32 (1:5000) [31] or anti-LRP antibody W3 (1:5000) [21]. Blots were then incubated with the secondary anti-mouse and anti-rabbit HRP conjugated antibody (Sigma) (1:5000), respectively, following detection using SuperSignal detection kit (Pierce).

2.4. Immune fluorescence and confocal microscopy

24-48 hours post transfection cells were fixed using 4% paraformaldehyd for 20 min at room temperature and were stained by 10 μg per ml DAPI for 10 min at room temperature. After threefold PBS wash cells were embedded using confocal matrix (MicroTechlab). The confocal analyses was carried out on a Leica DM IRE2 confocal microscope using the Leica confocal software TCS SP2 version 2.5.

3. Results

3.1. Cloning and Expression of the fluorescence labeled prion protein and the 37 kDa/67 kDa laminin receptor

The fluorescent fusion proteins were generated on the basis of pEGFP-N1 and pDsRed-N1 as well as pECFP-C1 and pDsRed-C1 vector backbones, respectively. The insertion resulted in fluorescence-coupled variants of the mouse prion protein and the murine laminin receptor fusion proteins (Fig. 1A). The enhanced green fluorescent protein (EGFP) was placed in frame between the prion protein-derived signal peptide [9] to obtain the EGFP-PrP fusion protein. The variant lacking the signal peptide was fused to the C-terminus of the DsRed sequence (DsRed- Δ SP-PrP). In case of the laminin receptor (LRP/LR) fusion proteins, the fluorescent proteins were fused in frame to the C-terminal end of LRP/LR to obtain LRP-ECFP, LRP-DsRed and LRP-EGFP, respectively. The expression of the prion protein fusion protein variants EGFP-PrP and DsRed- Δ SP-PrP in BHK cells was confirmed by Western Blotting, showing that both proteins were present in cell lysates (Fig. 1B, left panel, lanes 1, 2). The EGFP-PrP was typically glycosylated showing a three band pattern between 48 kDa and 60 kDa (Fig. 1B, left panel, lane 1), whereas the variant lacking the signal peptide was unglycosylated (Fig. 1B, left panel, lane 2). The increased molecular weight of the PrP proteins resulted from the EGFP fusion that has a molecular weight of approximately 35 kDa. Moreover, the specificity of the antibody stain (A7) was confirmed using non transfected control cell lysate (Fig. 1B, left panel, lane 3). In addition, the expression of the LRP/LR fusion proteins LRP-ECFP (Fig. 1B, right panel, lane 1), LRP-DsRed (Fig. 1B, right panel, lane 5) and LRP-EGFP (Fig. 1B, right panel, lane 3), respectively, have been verified employing the polyclonal LRP antibody W3 [25, 32].

3.2. EGFP-PrP accumulates in the perinuclear compartment

EGFP-PrP was transfected into BHK cells and subcellular localization was examined 24h post transfection by confocal fluorescence microscopy. In cells expressing high levels of the transgene an accumulation of EGFP-PrP in an endosomal compartment (Fig. 2A) which was localized in close proximity to the nucleus was observed (Fig. 2B, C). This finding is supported by previous publications demonstrating that EGFP-PrP is localized in either Golgi compartments or, after internalization of PrP^c, in endosomal compartments including an accumulation in the perinuclear compartment [16, 17].

3.3. DsRed-DSP-PrP is localized in the nucleus and does not colocalize with heterochromatin

The mutant PrP DsRed- Δ SP-PrP lacking the signal peptide revealed a distinct localization pattern (Fig 2B) in neuronal cells (N2a). The mutant PrP variant did not colocalize with EGFP-PrP (data not shown) and was localized in the nucleus and not in the cytoplasm (Fig 3 A). The nucleic localization of PrP lacking its signal peptide has been reported previously in a study using antibody-based fluorescent-staining [18]. Here, we confirm this finding by transient transfection of DsRed- Δ SP-PrP in BHK cells (data not shown) in addition to N2a cells (Fig. 3). However, upon co-staining of the DNA with DAPI (Fig. 3B, E) no colocalization of red fluorescent DsRed- Δ SP-PrP and the heterochromatin (Fig. 3C) was detected. Due to the indistinct analysis of the merge of the blue DAPI stain and the red fluorescence of DsRed- Δ SP-PrP, the filter of the confocal microscope was changed from blue to green for the visualization of DAPI (Fig. 3E, F). Our finding implicates that the herein used DsRed- Δ SP-PrP does not colocalize with heterochromatin.

3.4. The 37 kDa/67 kDa laminin receptor colocalizes with EGFP-PrP and partly colocalizes with DsRed-DSP-PrP in the nucleus

Furthermore, we investigated the distribution of the 37 kDa/67 kDa laminin receptor (LRP/LR) upon cotransfection using the different PrP variants. Cotransfection of EGFP-PrP and LRP-DsRed resulted in a colocalization in the perinuclear compartment and on the cell surface (Fig. 4A). In earlier studies we showed that PrP^c and LRP/LR colocalize on the cell surface of neuroblastoma cells [21]. In addition, LRP-ECFP also localized in the nucleus of cotransfected DsRed- Δ SP-PrP cells. This finding argues that upon expression of the PrP mutant, LRP/LR was partly directed to or localized to the nucleus (Fig. 4B) and not localizing within the perinuclear compartment. Previously, it has been shown that LRP/LR is widely spread through all cellular compartments including the cytoplasm, the nucleus and the cell surface where it contributes to its multifunctional role in cell biology [33-35]. Although a typical co-localization pattern was not observed, we found that LRP-ECFP and DsRed- Δ SP-PrP were partly colocalized in the nucleus (Fig. 4B).

4. Discussion

4.1. Putative role of the prion protein mutant DsRed-DSP-PrP in the nucleus

The eukaryotic cell nucleus is organized within subnuclear compartments including the nucleic envelope, the eu- and heterochromatin as well as the nucleoli as sites of ribosome production and assembly. A variety of proteins are localized in the nucleus and are part of the ribosomal, replication or transcription machinery and the spliceosome, respectively. However, a putative role of a prion protein located in the nucleus, in case its signal peptide is genetically removed, is highly speculative. So far, there is no evidence that PrP lacking its signal peptide is present in the nucleus under physiological conditions. Nevertheless, the nuclear localization of DsRed- Δ SP-PrP is astonishing. As it was previously discussed, there is no evidence, that a nuclear localization signal is responsible for such action [18]. Until now it is speculative how PrP in the absence of its signal peptide targets the nucleus. Further analyses are required to unveil this mode of action.

4.2. The 37 kDa/67 kDa laminin receptor and the prion protein co-localize intracellularly

In the present report, we show for the first time the intracellular co-localization of LRP/LR with PrP. We hypothesize that the nucleic localization of the mutant PrP might be based on the passive transport via an interaction with another protein. Such an interaction partner might be the the 37 kDa/67 kDa laminin receptor LRP/LR, which has been identified as the cell surface receptor for the cellular PrP and infectious prions [21-23]. To investigate this possibility, we generated fluorescent LRP variants, which were used for colocalization studies with both EGFP-PrP and DsRed- Δ SP-PrP. Our results are consistent with the wide cellular localization pattern of LRP-EGFP including the nucleus, the cytoplasm and the cell surface. Previously, it has been reported that LRP/LR, which is basically identical with the ribosomal protein p40 playing an important role in the translation process, is transported into the nucleus under specialized conditions [36]. It has further been shown that LRP/LR is located inside the nucleus, beside cytoplasmic and cell surface localization, where it binds to histones [34, 35]. Further experiments are required to enlighten the unknown mechanism by which cytoplasmic PrP variants are targeted to the nucleus.

Since internal nuclear localization signals are not involved in nucleic import of the prion protein lacking the signal peptide [18], an alternative way of nuclear import may involve an adapter protein that contains a nuclear localization signal. It has been shown that the 37 kDa

LRP is directed to the cellular nucleus after addition of midkine, a heparin binding growth factor [36, 37]. According to the binding ability of PrP to LRP [21, 38], we speculate that the nuclear localization of the PrP variant DsRed- Δ SP-PrP might be also dependent on midkine. This explanation, as well as the multifunctional role of LRP/LR in the cell, may account for our finding that depletion of LRP/LR does not totally impede nucleic transport of the mutant PrP. This issue shall be further addressed in cell biology studies to unravel the yet unidentified mode of action by which mutant PrP is transported into the nucleus.

4.3. Subcellular Localization of the mutant prion protein lacking the signal peptide

Previously, Crozet and colleagues described a chromatin association for the prion protein lacking its signal peptide [18]. However, the chromatin-removal method has some limitations which might not reveal reliable information on localization in vivo [39]. Here, we performed an additional colocalization experiment by the use of the DNA stain DAPI to confirm the chromatin association of mutant PrP. Astonishingly, we could barely find a co-localization of DAPI stained DNA with DsRed- Δ SP-PrP. This finding clearly argues against a tight chromatin binding of DsRed- Δ SP-PrP, because the distance of two molecules lacking co-localization clearly exceeds 200 nm in the x/y plane and even more than 400 nm in the z plane. The mutant protein is found in nucleic compartments containing a low DNA content, which may be nucleoli structures. Therefore, further experiments, in which subnuclear structures are specifically co-stained will be needed to investigate the subnuclear localization of DsRed- Δ SP-PrP.

In summary, we have confirmed, that EGFP-PrP is localized at the cell surface and in a perinuclear compartment within the cell and that mutant DsRed- Δ SP-PrP localized to the nucleus. Interestingly, we found that the latter PrP mutant failed to be co-localized with DAPI stained heterochromatin. Furthermore, in the present study we showed for the first time that (i) PrP-EGFP and LRP-DsRed co-localize in the perinuclear compartment in addition to a cell surface-co-localization and (ii) DsRed- Δ SP-PrP and LRP-ECFP partly co-localize in the nucleus, respectively. Thus, the 37 kDa/67 kDa LRP/LR and the prion protein reveal close proximity not only on the surface of eukaryotic cells but additionally in intracellular compartments. Investigations by immunoprecipitation or cellular fractionation will further unravel the role of PrP, PrP mutants and LRP/LR in the life cycle of prions.

Acknowledgements

We thank Dr. Kil Sun Lee from Brazil for the kind gift of the pEGFP-PrP plasmid. The work was supported by the Bundesministerium für Bildung und Forschung (grant 01-KO-0514), the European Commission (grant NoE-NeuroPrion FOOD-CT 2004-506579) and the Deutsche Forschungsgemeinschaft (grant WE 2664/2-1).

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Figure legends

Fig. 1. Expression of the PrP fusion proteins and the 37 kDa/67 kDa laminin receptor in mammalian cells. (A) Fluorescent gene fusion constructs were generated and transiently transfected into BHK cells to investigate the subcellular localization of PrP and LRP/LR, respectively. The cDNA for the signal peptide (SP) of murine PrP^c was cloned N-terminally, the cDNA encoding amino acids 23-254 C-terminally to EGFP and DsRed. The sequence of murine LRP was cloned N-terminally to the ECFP, DsRed and EGFP proteins, respectively. (B) Expression of PrP derived fusion proteins was analyzed 48 h post transfection (left panel). Both EGFP-PrP (lane 1) and DsRed-DSP-PrP (lane 2) in comparison to non-transfected cells (lane 3) were detected using the PrP-specific mAb SAF 32. Expression of LRP derived fusion proteins was analyzed in BHK cells 48 h post transfection (right panel). LRP-ECFP derived cell lysate (lane 1) and LRP-DsRed derived cell lysate (lane 3) were compared to mock-transfected cells (lanes 2, 4, 6) using the polyclonal anti-LRP serum W3.

Fig. 2. Localization of green fluorescent protein coupled prion protein (EGFP-PrP) in a perinuclear compartment. (A) EGFP-PrP was examined 48 h post transfection in BHK cells using confocal microscopy. (B) costaining of the nucleic lamina with a lamin B-specific antibody followed by incubation with a secondary antibody conjugated with indocarbocyanine Cy3. (C) merge of A and B. The bar represents 4 μm .

Fig. 3. The prion protein mutant DsRed- Δ SP-PrP does not colocalize with heterochromatin in the nucleus. (A) N2a cells were transiently transfected with DsRed- Δ SP-PrP and analyzed by confocal microscopy. Z sections through an individual cell were performed varying about 2.8 μm in the Z plane in total (individual steps 0.2 μm). (B) The same cell was simultaneously stained by DAPI (10 $\mu\text{g/ml}$) and analyzed for heterochromatin/ DNA staining, respectively. (C) Merge of A and B. The bar represents 6 μm . (D) N2a cells were transfected with DsRed- Δ SP-PrP and analyzed by confocal microscopy. (E) Simultaneously, the same cell was stained with DAPI (10 $\mu\text{g/ml}$) and analyzed for heterochromatin/DNA staining using the EGFP channel for better visualization. (F) Channels were merged. The bar represents 8 μm .

Fig. 4. Colocalization of the 37 kDa/67 kDa laminin receptor (LRP/LR) with green fluorescent protein coupled prion protein (EGFP-PrP) and in part-colocalization with the mutant prion protein DsRed- Δ SP-PrP. (A) BHK cells were transiently cotransfected with pEGFP-PrP and pLRP-DsRed. 48 hours post transfection cells were fixed and the distribution of fluorescent proteins was analyzed by confocal microscopy. Green channel (left panel), red channel (middle panel) and merge (right panel). (B) BHK cells were transiently cotransfected with pDsRed- Δ SP-PrP and pLRP-ECFP. 48 hours post transfection cells were fixed and the distribution of fluorescent proteins was analyzed by confocal microscopy. Red channel (left panel), blue channel (middle panel) and merge (right panel). The bar represents 20 μ m.

Figure 1

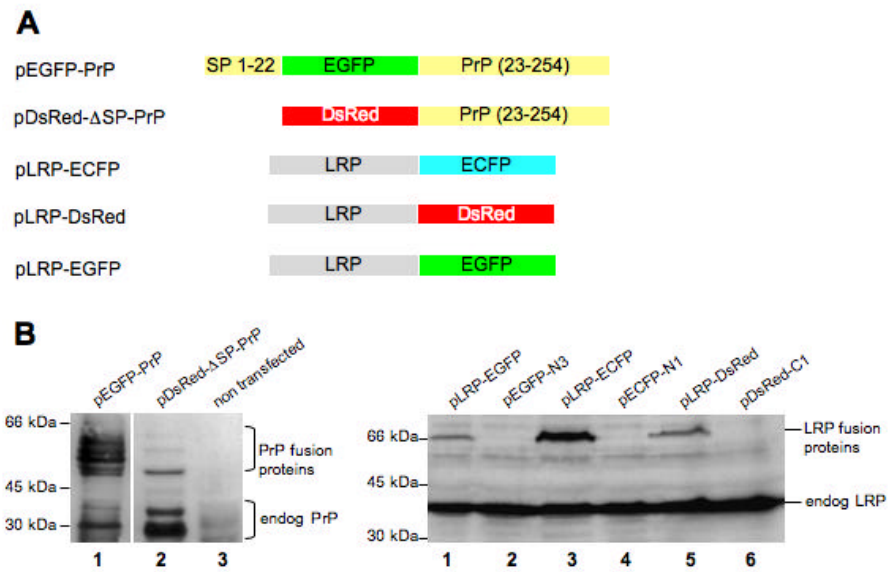
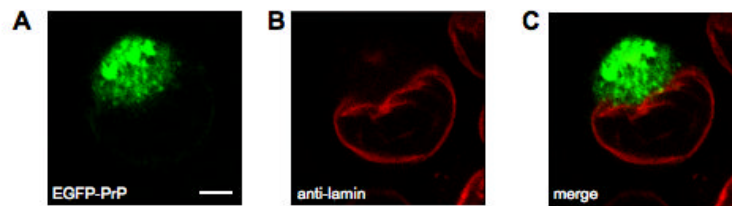


Figure 2



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Figure 3

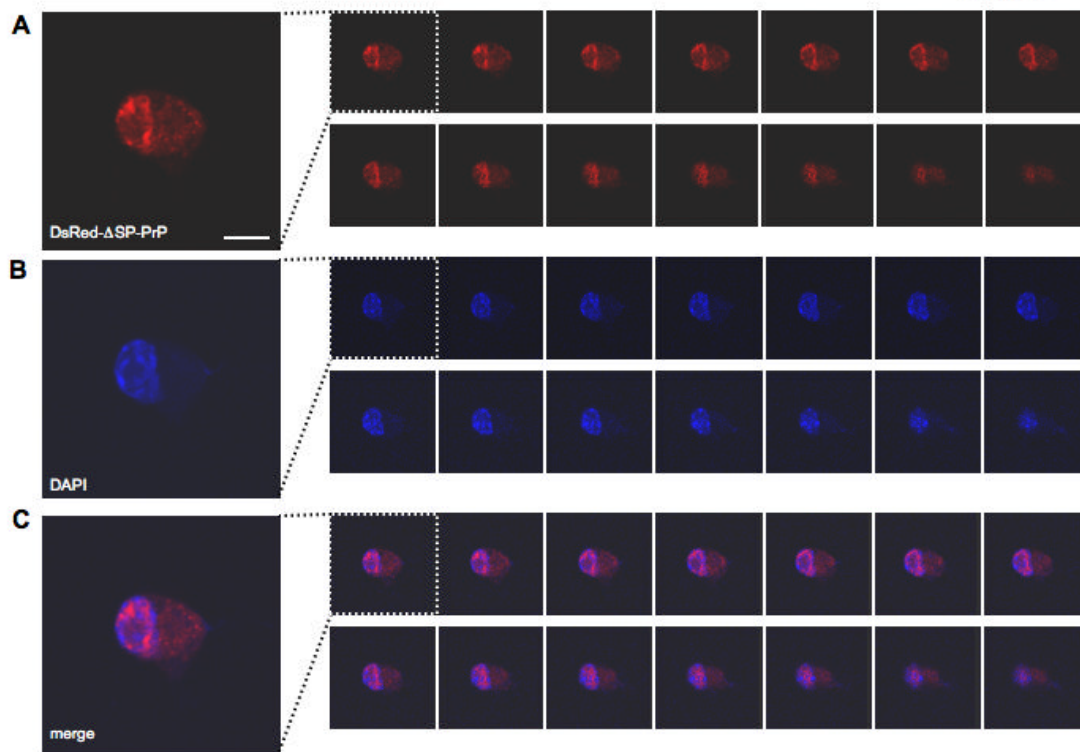
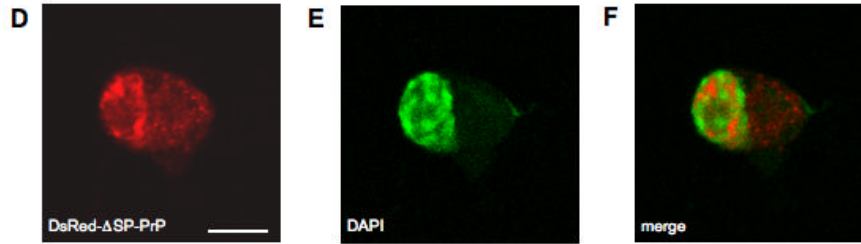
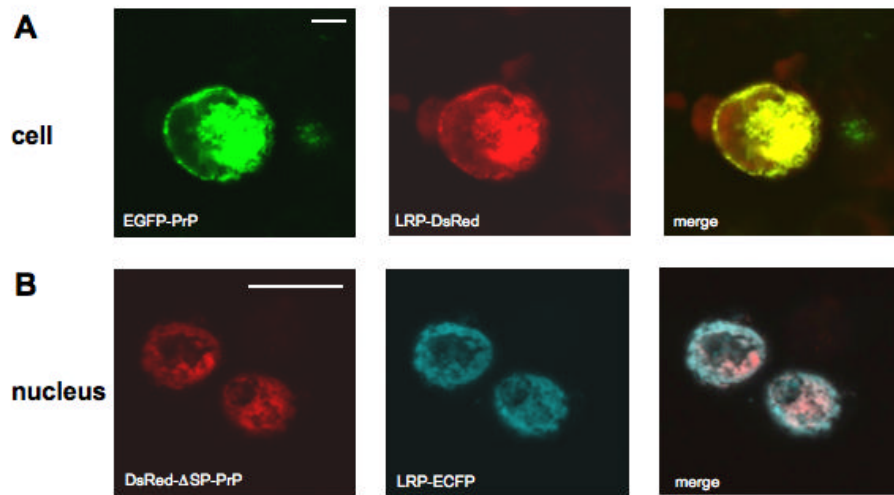


Figure 3



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Figure 4



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