

Examination of the effect of a downstream translation initiation site on the localization of proteins using the secretory pathway

Barbara Vodicska¹, Antal Nyeste², Eszter Tóth³, Ervin Welker²

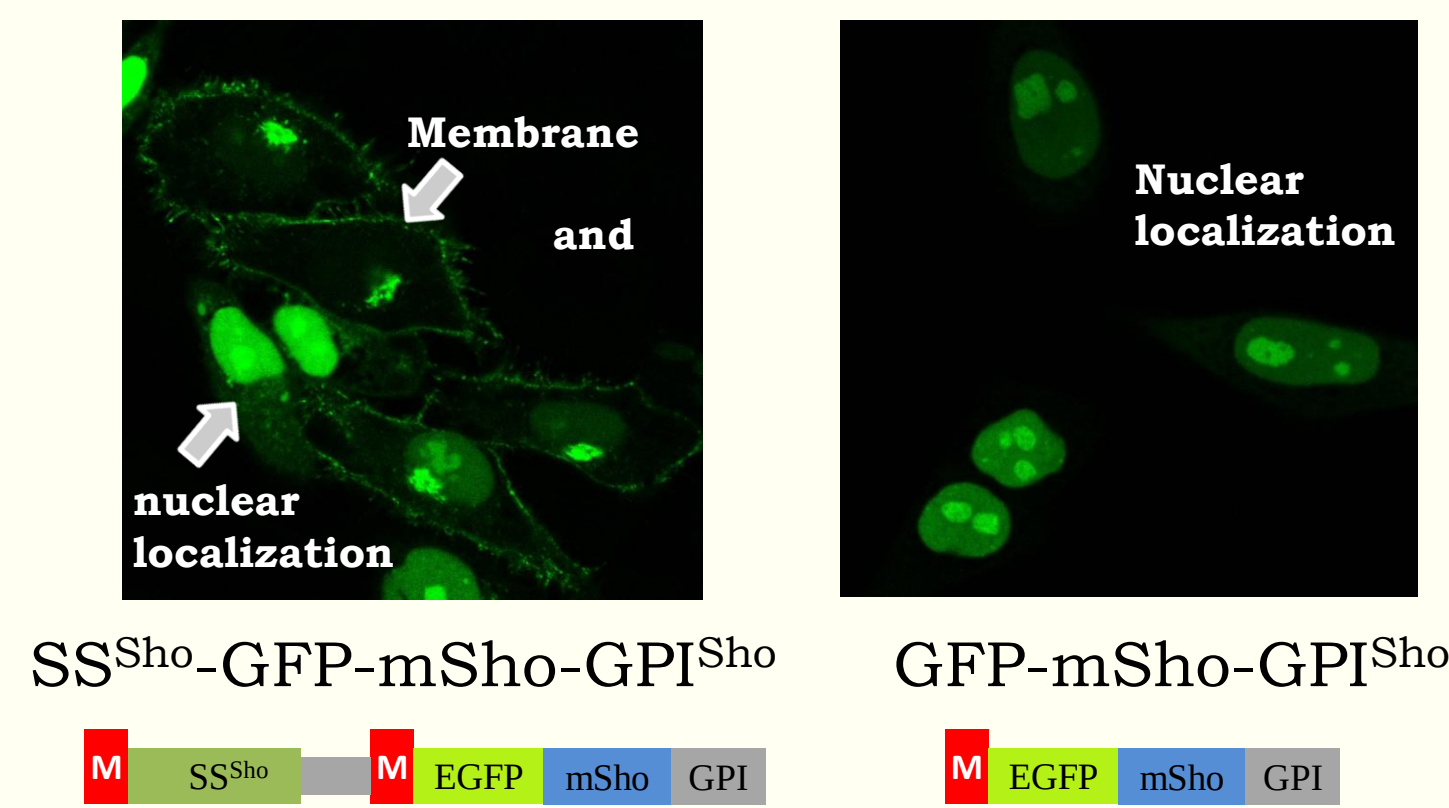
¹ Department of Applied Biotechnology, Faculty of Chemical Technology and Biotechnology, Budapest University of Technology and Economics
² Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences
³ Department of Anatomy, Cell and Developmental Biology, Institute of Biology, Faculty of Science, Eötvös Loránd University

Introduction

Fluorescent proteins are often used for studying protein localization and trafficking. When they are fused to the N-terminus of proteins using the secretory pathway, they must be inserted after the secretion signal. It is a common practice to insert the fluorescent protein in a way which **leaves the starting translation initiation (TI) site and the associated Kozak sequence⁽¹⁾ of the fluorescent protein intact**. This approach is **based on the ribosome scanning model** of TI according which the translation starts practically exclusive at the first initiation site, even if there are more downstream ones ^(1, 2, 3). Using this cloning strategy, the **Shadoo protein** (a member

of the prion protein family that are membrane proteins and use the secretory pathway) was **found in the nucleus** of a fraction of the cells. **We aim to understand the origin of this dual localization** by designing a construct that facilitates to distinguish between the products of the first and the second TI site. The effect of mutating the second downstream TI site, and of inserting out-of-frame TI sites into the signal peptide (SS) in other constructs, is examined by observing the ratio of cells showing nuclear YFP (yellow fluorescent protein) signal between the product of the first and second TI.

The Shadoo protein in an unnatural localization: **in the nucleus**



Materials and methods

Rationale:

- Translation from the **1st AUG**: The protein goes to the secretory pathway and localizes in the **plasma membrane** with a GPI-anchor.
- Translation from the **2nd AUG**: No secretion signal (SS) cytosolic expression. Thus, due to a nuclear localization signal⁽⁴⁾ (NLS) it localizes in the **nucleus**.

Additional methionine constructs:

We inserted out-of-frame TI sites (including an ATG triplet and the surrounding Kozak sequence) in two positions of the SS: right after the starting ATG and some nucleotides before the second one. In case of leaky scanning we expect these might hijack the leaky ribosomes reading through the first AUG by initiating the expression of completely different

polypeptides and, in turn, decrease the number of proteins expressed from the second TI site.

Statistics:

All the constructions were transfected in **HeLa cell line**. The transfected cells were examined using laser scanning confocal microscopy with Hoescht33342 used, as nuclear staining.

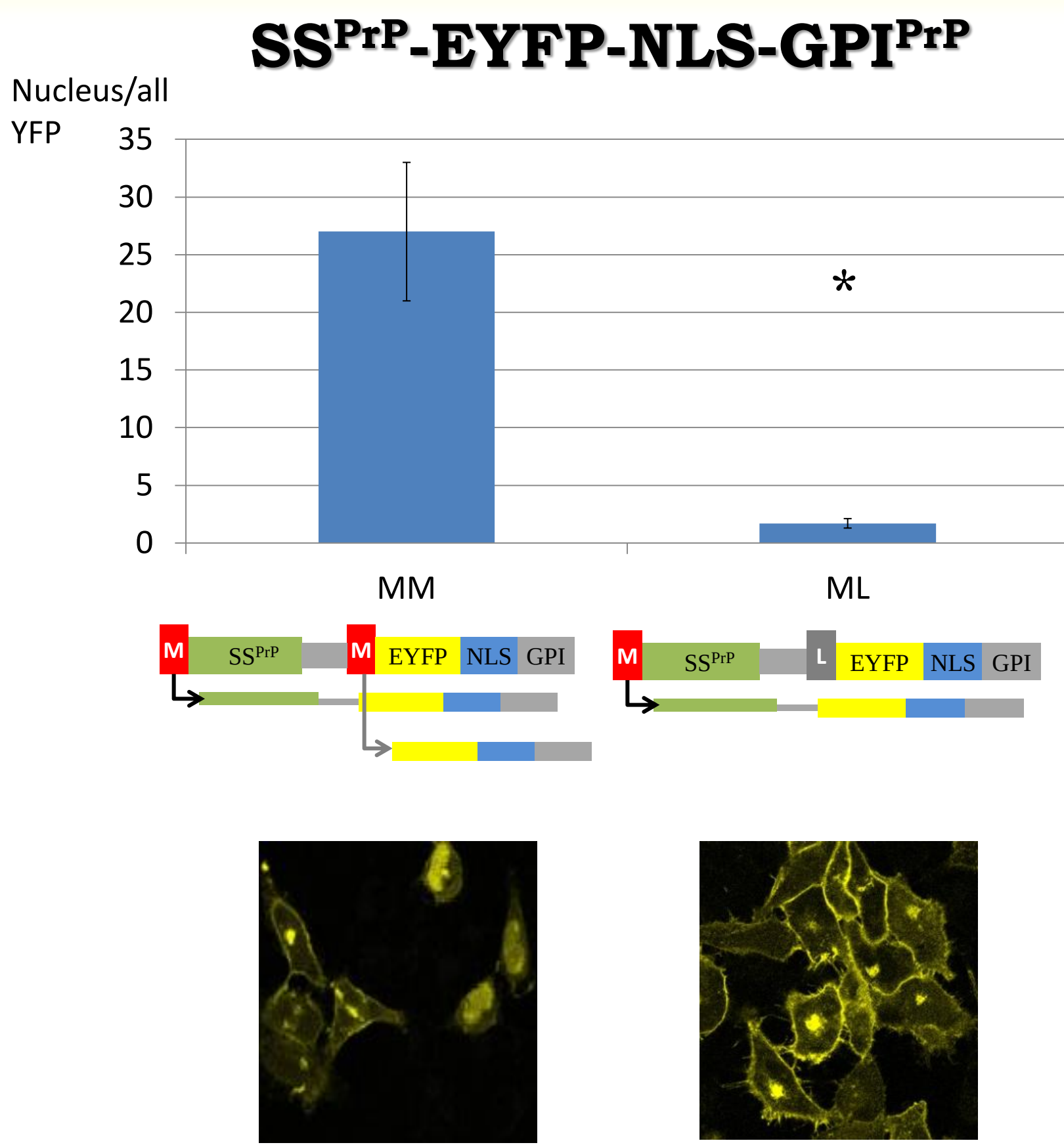
In each experiment, the nuclear and plasma membrane localizations were counted in at least 100 cells in each 3 or 4 parallel experiments. The Student's t-test or the one-way ANOVA test (with Dunnett post-hoc test) was used to check significances.

Symbols on the graphs: * p<0.05, ** p<0.01, *** p<0.001 - compared to the MM control and # p<0.05 - compared to the M(Li)M control.

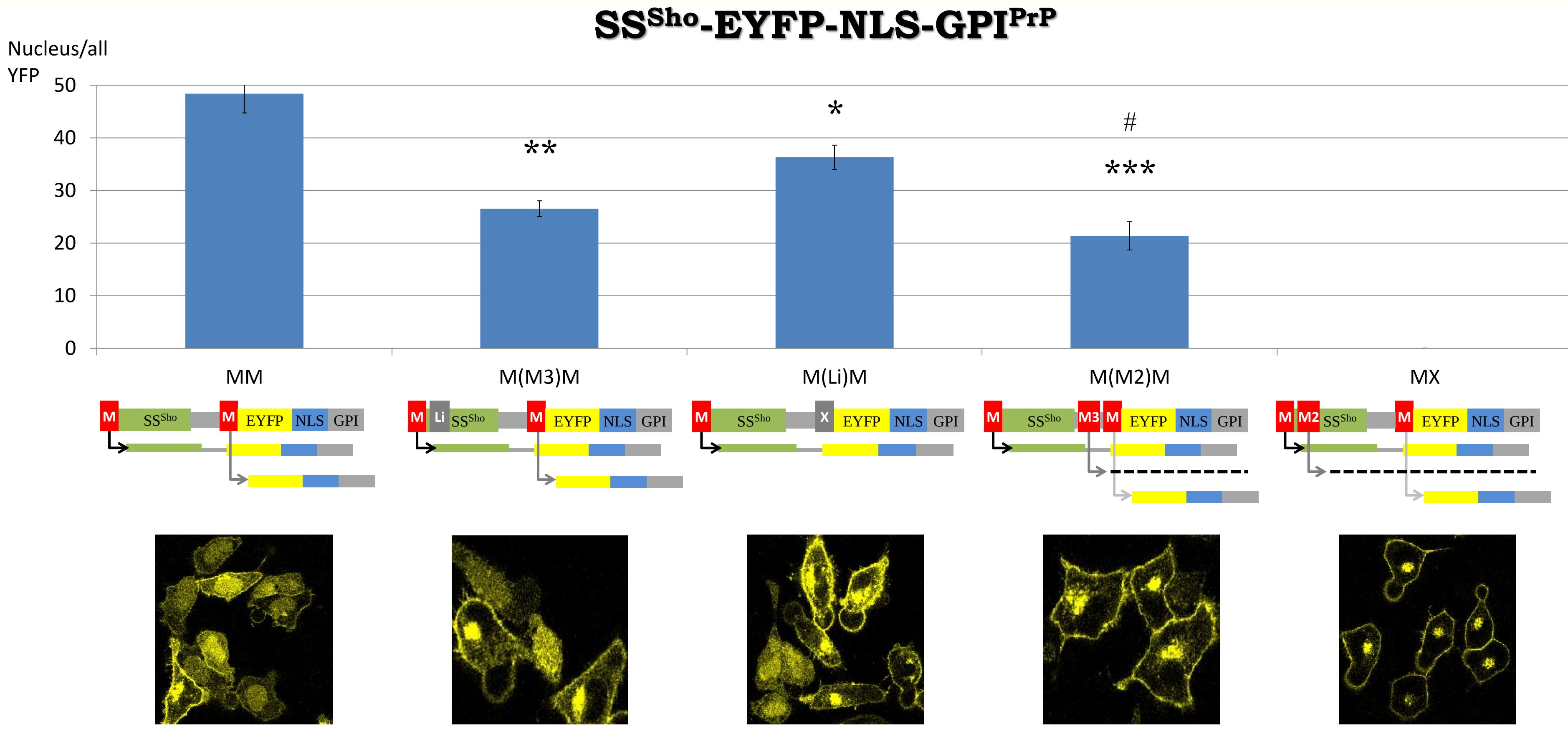
Results

In the presence of both TI sites (MM) we observed **nuclear localization** in nearly 30% of the transfected cells, which was **almost entirely eliminated when the downstream TI site was mutated** to TTG (ML) - (The SS from the PrP - prion protein.)

Inserting out-of-frame TI sites also decreased the nuclear localization ratio - (The SS from the Sho - Shadoo protein.) Insertion of an additional **out of frame TI site just preceding the second one (M(M3)M) resulted in a 20% decrease** in the nuclear localization compared to the double TI site (MM) construct.



Symbols: MM – TI sites in the beginning of both the SS and YFP, ML – the second TI site mutated to leucine (potential alternative TI site according to Kozak ⁽¹⁾ – TTG)



Symbols: MM – TI sites in the beginning of both the SS and the YFP, M(M3)M – double TI site and an additional out-of-frame TI site inserted after the SS, M(M2)M – double TI site and an additional TI site right after the first one, M(Li)M – double TI site plus an M2-similar amino acid coding oligonucleotide, MX – the second TI site mutated.

References

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- (2) Translation of insulin-related polypeptides from messenger RNAs with tandemly reiterated copies of the ribosome binding site. **M. Kozak**. Cell 34 (1983) 971-8.
- (3) The scanning model for translation: an update. **M. Kozak**. The Journal of Cell Biology, 108 (1989) 229-241.
- (4) The Quaking I-5 protein (QKI-5) has a novel nuclear localization signal and shuttles between the Nucleus and the Cytoplasm. **Jiang Wu, Li Zhou, Kathryn Tonissen, Ronald Tee, and Karen Artzt**. The Journal of Biological Chemistry. 274, 41. (1999) 29202-29210.
- (5) Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes. **M Kozak**. Proc. Natl. Acad. Sci. USA, 87 (1990) 8301-8305.

Summary and Conclusions

In conclusion, when the trafficking and localization of ER-targeted proteins are monitored by the use of an N-terminal fluorescent protein tag, the original starting **TI site of the fluorescent protein has to be eliminated in order to avoid the appearance of a truncated fusion protein** that is directed to the cytosol. This might lead to **unnatural localization** and **decrease the signal : background ratio in fluorescent imaging**.

Having been examined with two kinds of secretion signal (SS^{Sho} and SS^{PrP}), the phenomenon we studied is **likely not associated with a specific SS**. These results are consistent with leaky ribosomal scanning, a form of alternative translation initiation occurring here; a phenomenon thought to be very rare. However, further experiments are necessary to exclude contributions from transcriptional level.