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# Engulfment adapter PTB domain containing 1 (GULP1) is a nucleocytoplasmic shuttling protein and transactivationally active together with lowdensity lipoprotein receptor-related protein 1 (LRP1)

**Anke Wahler**[*,#], **Anja-Silke Beyer**[*,#], **Ilona E. Keller**[*,#], **Cathrin Schnack**[*], **Björn vonEinem**[*], **Christian Pröpper**[†], **Tobias M. Boeckers**[†], **Ithan D. Peltan**[‡], **Dudley K. Strickland**[§], **Bradley T. Hyman**[‡], and **Christine A. F. vonArnim**[*]

[*]Department of Neurology, Ulm University, 89081 Ulm, Germany

[†]Institute of Anatomy and Cell Biology, Ulm University, 89081 Ulm, Germany

[‡]Alzheimer's Disease Research Laboratory, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA

[§]Center for Vascular and Inflammatory Diseases, University of Maryland School of Medicine, Baltimore, MD 21201, USA

## Abstract

Amyloid-β (A4) precursor protein (APP) and low density lipoprotein receptor-related protein 1 (LRP1) have been implicated in pathogenesis of Alzheimer's disease (AD). They are functionally linked by Fe65, a phosphotyrosine binding domain (PTB) domain containing adaptor protein that binds to intracellular NPxY-motifs of APP and LRP1, thereby influencing expression levels, cellular trafficking and processing. Additionally, Fe65 has been reported to mediate nuclear signaling in combination with intracellular domains of APP and LRP1. We have previously identified another adaptor protein, engulfment adapter PTB domain containing 1 (GULP1). Here, we characterize and compare nuclear trafficking and transactivation of GULP1 and Fe65 together with APP and LRP1 and report differential nuclear trafficking of adaptors when APP or LRP1 are co-expressed. Observed effects are additionally supported by a reporter plasmid based transactivation assay. Our data indicate that Fe65 might have signaling properties together with APP and LRP1, whereas GULP1 mediates only LRP1 transactivation.

### Keywords

adaptor proteins; signaling; transactivation; amyloid precursor protein



**To whom correspondence should be addressed:** C.A.F. von Arnim, Dept. of Neurology, UlmUniversity, Helmholtzstr. 8/1, 89081 Ulm, Germany, christine.arnim@uni-ulm.de, FAX: +49 731 177 1202.
[#]authors contributed equally.

**DISCLOSURE STATEMENT**
The authors disclose that there are no actual or potential conflicts of interest.

## INTRODUCTION

Alzheimer's disease (AD) is histopathologically characterized by neurofibrillary tangles and senile plaques consisting of amyloid-β (Aβ). AD has been genetically linked to amyloid-β (A4) precursor protein (APP) [1]. Interaction of APP with other proteins, including the low density lipoprotein receptor related protein 1 (LRP1) and Fe65, contribute to AD pathology[2-3]. These three proteins form a tripartite complex in which Fe65 links the intracellular tails of APP and LRP1 [4].

Both APP and LRP1 are type I transmembrane proteins with a large ectodomain and a short cytoplasmic tail. There are several human APP isoforms of differing lengths, the shortest form (APP695) is predominantly expressed in neurons. Differential processing of APP by α-/γ- or β-/γ-secretases leads to distinct products with the latter processing pathway leading to generation of Aβ [5]. Both pathways, however, result in generation of APP intracellular domain (AICD), which has been implicated in signal transduction together with its adaptor Fe65 and Tip60[6].

The mature LRP1 molecule has an 85 kDa light chain (LRP1-LC) that contains the transmembrane domain and the cytoplasmic tail by which LRP1 interacts with multiple intracellular adaptor and scaffold proteins [7-8], and a large 515 kDa heavy chain that contains multiple ligand binding domains. LRP1 mediates endocytosis of multiple ligands, including several of potential importance for Alzheimer pathophysiology: APP, Aβ, apolipoprotein E and $\alpha_2$-macroglobulin [7-9].

LRP1 has recently been shown to be a γ-secretase substrate. Cleavage releases the LRP1 intracellular domain (LICD), which can translocate to the nucleus and interact with Tip60 [10-11]. Furthermore, LICD binds and promotes the nuclear export and proteosomal degradation of interferon regulatory factor 3 [12].

The cytoplasmic tails of APP or LRP1 contain one (APP) or two (LRP1) conserved NPxY-motifs. NPxY-motifs are important as a signal for clathrin-coated pit internalization and also serve as docking sites for proteins encoding a phosphotyrosine-binding (PTB) domain, which include Dab1, Fe65, JIP-1b, Numb, Shc and X11/MINT [13]. APP and LRP1 are linked by Fe65, an adaptor protein containing two PTB domains. The proximal PTB domain binds to APP's NPxY-motif [14], while the distal PTB domain of Fe65 can interact with one or both LRP1 NPxY-motifs [4, 15]. We and others have recently identified a novel intracellular APP adaptor protein, engulfment adapter phosphotyrosine binding domain containing 1 (GULP1) [16-17], the human homologue of *C. elegans* death (CED)-6, whose function in engulfment is highly conserved among species. GULP1 acts as a promoter of phagocytosis in human macrophages and might function as a signaling adaptor downstream of CED-1. Accordingly, the PTB domain of GULP1 has recently been shown to interact with the second NPxY motif in the intracellular tail of LRP1, the presumed human homologue of CED-1 [18-20].

In this study, we compare the signaling properties of the adaptor proteins Fe65 and GULP1 by comparing transactivation capabilities of APP's or LRP1's intracellular domains (AICD/LICD) as well as of GULP1 or Fe65 in a reporter gene-based assay. Further, we demonstrate that the cellular distribution and nuclear trafficking of nucleocytoplasmic shuttling proteins GULP1 and Fe65 are altered by co-expression of the neuronal APP695 isoform or LRP1's light chain (LRP1-LC) in Neuro-2A cells.

# MATERIALS AND METHODS

## Antibodies and reagents

Antibodies directed against the hemagglutinin (HA)- or myc-tag (9E10) were purchased from Sigma (Munich, Germany). Gene-directed antibodies were against APP N-terminus (22c11, Millipore, Billerica, MA, USA) or C-terminus (Sigma), Gal4, LRP1-LC, GULP1 (Santa Cruz Inc., CA, USA), MEK1/2 (47E6, Cell Signaling Technology, Danvers, MA, USA), p84 (5E10, Abcam, Cambridge, MA, USA). The antibody against Fe65 was kindly provided by C. Pietrzik (Mainz, Germany) [4]. Secondary fluorescent dye (Alexa-488/ Alexa-546) or horseradish peroxidase coupled antibodies were obtained from Molecular Probes/Invitrogen (Paisley, UK). Leptomycin B (LMB) was obtained from AppliChem (Darmstadt, Germany).

## Generation of expression constructs

An overview of all protein constructs used in this study can be found in Fig. 1.

Expression vectors for human APP695-myc and its Y682/687A-mutant were described previously [16] as well as myc-LRP1-LC [21]. Mutations in the second NPxY-motif sequence of LRP1-LC-myc were introduced with the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), changing the $^{4504}$NPVY$^{4507}$-sequence to $^{4504}$APVA$^{4507}$.

Fe65-HA was generated by PCR using human Fe65-Flag as a template, which was generously supplied by C. Pietrzik, and primers introducing restriction sites for KpnI (5′GCGGTACCATGTCTGTTCCATC3′) and BamHI (5′GTAGGATCCTGGGGTATGGGC3′) for cloning it in frame into phCMV3 vector (Genlantis). The construction of GULP1-HA (aa1-304), PTB-HA (aa1-168) and ΔPTB-HA (aa169-304) was described previously [16].

Plasmid constructs for the transactivation assays encoding Gal4-fusion proteins were APP-Gal4 [6] (kind gift of T. Sudhof) and LRP1-LC-Gal4/VP16 [21]. Additionally, mutations in the $^{682}$YENPTY$^{687}$-motif of APP-Gal4 to $^{682}$AENPTA$^{687}$ and the second NPxY-motif sequence of LRP1-LC-Gal4/VP16 were introduced by site directed mutagenesis. Gal4-GULP1 was constructed by deletion of the APP sequence N-terminal of Gal4 in the APP-Gal4 vector with NheI and in frame insertion of PCR amplified GULP1 C-terminal of Gal4 with primers introducing restriction sites for EcoRI (5′GGGGAATTCGTCGACATGAACCGTGCTTTTAG3′) or stop-codon and BamHI (5′AATGGATCCTTAGCACCTACTGTCTAACGGTCGAGACA3′). Empty pMST control vector was generated by cutting out GULP1 sequence with SalI. Fe65-Gal4 was generated by PCR using primers introducing NheI restriction sites (5′AAAGCTAGCATGTCTGTTCCATCATCA3′, 5′AAAGCTAGCCCATTCAAGTCCTCTTCA3′) and cloning it in frame into empty pMST-vector. Reporter plasmid pG5E1B encoding luciferase under control of a Gal4-dependent promoter was also a kind gift of T. Sudhof[6]. A plasmid containing the sequence of β-Galactosidase was obtained from Stratagene (La Jolla, CA, USA).

## Cell line and transfection

Murine neuroblastoma Neuro-2A cells were cultured under standard cell culture conditions in DMEM (Gibco/Invitrogen) containing 10% FBS Gold (PAA, Pasching, Austria) and 1x penicillin/streptomycin. Transient transfections were performed using SatisFection transfection reagent (Stratagene, La Jolla, CA, USA) following manufacturer's instructions. For siRNA knockdown experiments using a human GULP1-specific package of preselected

siRNAs or Alexa-488 coupled negative control siRNA(Qiagen, Hilden, Germany), HEK293 cells were transfected with Lipofectamine2000 (Invitrogen). Efficiency of knockdown was determined by immunoblotting.

### Immunocytochemistry (ICC)

Neuro-2A cells were grown on glass slides and transfected at low cell density. 24 hours after transfection, cells were fixed in 4% PFA/Sucrose and stained as described in [16]. Designated samples underwent LMB treatment (5ng/ml) for 2h prior to fixation.

To detect the localization of endogenous GULP1, Neuro-2A cells grown on glass slides were fixed and immunostained with a GULP1 directed antibody (Santa Cruz Inc., CA, USA).

### Confocal laser scanning microscopy (CLSM)

Confocal images of transfected or immunostained cells were recorded with a Zeiss LSM 710 Laser scanning microscope (Carl Zeiss, Jena, Germany) as previously described [16].

### Nuclear Extraction Assay

Neuro-2A cells were grown until 60-70% confluency and transfected with GULP1 or empty vector. 48 hours after transfection, cells were harvested in 1 ml PBS and centrifuged for 5 min at 510 *g*. Cells were resuspended in sucrose buffer (320mM Sucrose, 10mM TrisHCl pH 8.0, 3mM $CaCl_2$, 2mM NaAc, 0.1mM EDTA, 0.1mM DTT) containing 0.5% Igepal CA-630 and centrifuged for 10 min at 510 *g* to pellet the nuclei. The supernatant was transferred to a new tube and centrifuged at 14.000 *g* for 15 min. The obtained supernatant represented the cytosolic fraction. The nuclear pellet was washed with sucrose buffer without Igepal CA-630 and centrifuged again for 5 min at 510 *g*. The buffer was removed thoroughly and the pellet was resuspended in low salt buffer (20mM Hepes pH 7.9, 1.5mM $MgCl_2$, 20mM KCl, 0.2mM EDTA, 25% glycerol (v/v), 0.5mM DTT). An equal volume of hypertonic high salt buffer (20mM Hepes pH 7.9, 1.5mM MgCl2, 800mM KCl, 0.2mM EDTA, 25% glycerol (v/v), 1% Igepal CA-630, 0.5mM DTT) was added very slowly to extract the nucleoplasm. The sample was incubated for 45 min at 4°C. The nuclear fractionwas centrifugated at 14.000 *g* for 15 min to clear the sample from nuclear debris. Samples were analyzed by immunoblotting. Purity of fractions was determined by detection of nuclear (p84) or cytosolic (MEK1/2) marker proteins.

### LDS-PAGE and immunoblotting

Protein samples were electrophoresed under denaturing conditions using NuPAGENovexprecast system from Invitrogen as previously described [16].

### Transactivation assay

Transactivation assays were performed in Neuro-2A cells. Cells were grown in 12-well plates and transfected fivefold with β-gal (0.1 μg DNA), pG5E1B-luc (0.25 μg), Gal4-construct (0.25 μg), adaptor(s) and/or empty vector (0.2 μg each). After 24 hours, cells were lysed in Reporter Lysis Buffer (Promega, Mannheim, Germany). Expression of adaptors was confirmed by immunoblotting. Luciferase was measured in duplicate with Beetle Long-Glow-Juice (PJK, Kleinbittersdorf, Germany). Values were normalized to β-gal activity. Since effects of the luciferase reporter assay are to some extent very low, overexpression was mandatory.

### Statistical analysis

For transactivation assays, data were tested for normality using Kolmogorov-Smirnov test, followed by a Mann-Whitney Rank sum test using SigmaStat 3.5. P-values are indicated as follows: **<0.01, ***<0.001. Data are representative of at least three independent experiments that yielded similar results.

## RESULTS

### Fe65 and GULP1 are nucleocytoplasmic shuttling proteins

As Fe65 was previously reported to be a nucleocytoplasmic shuttling protein[22], we investigated whether GULP1 might have the same properties. To detect the cellular distribution of Fe65 and GULP1, confocal images were acquired using cells transfected with HA-tagged adaptor proteins. As nucleocytoplasmic shuttling is a rapid process, designated samples were treated with nuclear export inhibitor LMB (5ng/ml, 2h) prior to fixation. LMB, a *Streptomyces spp.* metabolite, is a useful tool to identify nucleocytoplasmic shuttling of proteins, as it blocks their nuclear export by binding to exportin 1/CRM1, a cargo protein for proteins containing a nuclear export signal (NES), resulting in accumulation of shuttling proteins in the nucleus. Since Fe65 but not GULP1encodes for a putative NLS and NES [22-23], one would expect, that LMB treatment would affect Fe65 and not GULP1 cellular localization.

When adaptors were overexpressed, Fe65localized both in the cytoplasm and in the nucleus, whereas GULP1 was found mostly in the cytoplasm and only to a small extent within the nucleus. However, upon LMB treatment, both Fe65 and GULP1 were enriched in the nucleus (Fig. 2A). To check physiological relevance of nucleocytoplasmic shuttling of GULP1, untransfectedcells were immunostained with a GULP1 specific antibody and analyzed by CLSM. ICC experiments revealed that endogenous GULP1 localized mainly in perinuclear region as previously been shown [16], but can also be found within the nucleus, even without LMB treatment (Fig. 2B).Quantitative analysis was carried out by counting cells of five nonoverlapping randomly choosen fields. Nearly 50% of all counted cells showed additionally nuclear localization of GULP1. Similar results were obtained using human embryonic kidney 293 cells or the human neuroblastoma cell line SH-SY5Y (data not shown). To investigate whether ICC results for GULP1 are consistent with another approach, a nuclear extraction assay was performed. Fe65 was used as control, since it is already known that Fe65 cycles between cytosolic and nuclear compartments[22, 24].Results showed that both HA-tagged Fe65 and GULP1 were detectable in the nucleus, even without LMB treatment. Furthermore, endogenous GULP1 was also found within the nuclear fraction. It seemed that LMB treatment had nearly no effect on nuclear shuttling of endogenous GULP1, whereas both overexpressed Fe65 and GULP1 were found enriched within the nucleus after LMB treatment (Fig. 2C).

### GULP1 mediates transactivation of LICD but not AICD

Prior studies have suggested that AICD and LICD function in regulating gene expression [6, 10-11, 21] and the role of Fe65 in this process has been confirmed [6]. To investigate whether GULP1 can influence translocation and transactivation of AICD and LICD a functional reporter gene-based transactivation assay was performed in which either Fe65 or GULP1 were co-expressed with fusion proteins of APP-Gal4 or LRP1-LC-Gal4/VP16. In this assay, γ-secretase processing releases the AICD or LICD, subsequent nuclear trafficking of AICD-Gal4 or LICD-Gal4 leads to activation of a Gal4-dependent promoter and transcription of the luciferase reporter gene.

All assays were performed in triplicate and repeated at least three times independently. Equal expression of constructs was verified by immunoblotting. In contrast to APP-Gal4, LRP1-LC-Gal4/VP16 encodes for the additional transcription enhancer VP16 leading to very high transactivation activity of the LRP1-LC-Gal4/VP16 construct itself [25].Our results, in line with previous experiments [6], demonstrated that Fe65 triggers APP-Gal4 transactivation to a greater extent than LRP1-LC-Gal4 [21]. GULP1, however, enhances LRP1-LC-Gal4 transactivation more than Fe65 (Fig. 3B) and has no effect on APP-Gal4 transactivation (Fig 3A). To test which domain of GULP1 is responsible for LRP transactivation we used deletion mutants showing that both the PTB-domain and Δ-PTB domain are necessary for transactivation. Interestingly, co-transfection of both adaptors caused suppression of LRP1-LC-Gal4 transactivation compared to GULP1 single transfection, but this effect was not significant.

Control experiments included Fe65 or GULP1 co-expressed with control Gal4 vector and/or luciferase reporter plasmid and showed no significant expression of luciferase, as did experiments with Gal4-fusion proteins containing mutations in the NPxY-motif of APP695 and LRP1-LC (data not shown).

## APP or LRP1-LC co-expression retains Fe65 and GULP1 in the cytosol and is dependent on the NPxY domain

Since both Fe65 and GULP1 impact APP695 and LRP1-LC transactivation, we investigated the influence of APP and LRP1 on nuclear trafficking of Fe65 and GULP1.

Therefore, ICC experiments were repeated with co-expression of HA-tagged Fe65 and GULP1 and myc-tagged APP or LRP1-LC.

Co-expression of APP695 altered the cellular distribution of both adaptors: Upon APP695 co-expression, Fe65 was held back in the cytosol andonly a minor amount was detectable in the nucleus. Furthermore, Fe65 almost completely co-localized with APP695 in perinuclear region (Fig. 4A, left panel, 1). In the case of GULP1, its expression also largely overlapped with APP695 (Fig. 4B, left panel, 1). In contrast to Fe65, GULP1 was nearly completely held back in the cytosol by APP695. To address whether APP695 might influence shuttling properties of these two adaptor proteins, experiments were repeated with LMB-administration. Interestingly, LMB treatment revealed that both Fe65 and GULP1 still managed to shuttle between nucleus and cytoplasm (Fig. 4A/B, right panel, 1) upon APP695 co-expression.

To test whether this effect was dependent on the YENPTY-motif, we transfected adaptors in combination with an APP-Y682/687A-mutant and found that both Fe65 (Fig. 4A, 2) and GULP1 (Fig. 4B, 2) resumed similar nucleocytoplasmic shuttling patterns as when they were overexpressed alone. Therefore, the PTB-domain/YENPTY-motif interaction was essential for observed effects. Other point mutations in APPs YENPTY-motif (N684A, P685A and Y687A) led to similar results (data not shown).

To explore whether LRP1-LC influence shuttling properties of its adaptors in a similar manner, we co-expressed LRP1-LC with either Fe65 or GULP1. The cellular distribution of these two adaptor proteins in combination with LRP1-LC was distinct from that observed with APP695: Fe65 co-localized with LRP1-LC but was not retained in the cytosol (Fig. 4A, left panel, 3). GULP1 also co-localized with LRP1-LC, but a small amount was still detectable within the nucleus (Fig. 4B, left panel, 3). However, LMB treatment confirmed that GULP1 was not completely retained in the cytosol and could be enriched in the nucleus (Fig. 4B, right panel, 3).

Upon co-expression of LRP1-LC-$N^{4504}A/Y^{4507}A$-mutant (mutations in the $2^{nd}$NPxY-motif) with Fe65 or GULP1, co-localization was diminished and the cellular distribution of the adaptors resembled that in cells transfected with the adaptor proteins alone (Fig. 4A/B, 4). This suggests that the $2^{nd}$NPxY-motif is essential for Fe65 and GULP1 interaction.

To further confirm the effect of APP695 and LRP1-LC on nucleocytoplasmic shuttling of GULP1, a nuclear extraction assay was performed. HA-tagged GULP1 was detectable in the nucleus, even upon APP695 or LRP1-LC co-expression. Moreover, LMB treatment caused further accumulation of overexpressed GULP1 within the nucleus (Fig. 4C).

### APP and LRP1-LC trigger transactivation of Fe65, while GULP1 transactivation is only enhanced upon LRP1-LC co-expression

Fusion proteins of Gal4 with Fe65 or GULP1 were constructed to test the transcriptional capability of both nucleocytoplasmic shuttling proteins themselves. Results revealed that Fe65-Gal4 co-expression with APP695 or LRP1-LC resulted in increased transcriptional activity compared to Fe65-Gal4 expression alone (Fig. 5A). Gal4-GULP1, by comparison, showed no transcriptional activity when transfected alone or in combination with APP695. However, co-expression of LRP1-LC led to increased transcriptional activity (Fig. 5B).

These results support the observations obtained by confocal imaging.

### The effect of GULP1 on LICD transactivation is reversible by GULP1 knockdown

Since overexpressed GULP1 enhances LRP1-LC-Gal4 transactivation we wanted to know whether this effect is suppressible by GULP1 knockdown. Therefore, HEK293 cells were transfected with GULP1-specific siRNAs or negative control siRNA. Efficiency of GULP1 knockdown was analysed by immunoblotting. siRNA-mediated knockdown reduced the endogenous GULP1 level by nearly 50%. Transactivation assays with overexpressed and knocked GULP1 were performed confirming our findings that overexpression of GULP1 stimulates LICD transactivation. On the other hand, knockdown of GULP1 leads to a significantly decreased tranactivational activity of LRP1-lc-Gal4 (Fig. 6). Interestingly, co-transfection of Fe65 showed no additve effect.

## DISCUSSION

### Nuclear trafficking of Fe65 and GULP1 is distinct

The function of proteins is dependent on their site of action. Here, we report that both Fe65 and GULP1 are nucleocytoplasmic shuttling proteins, a process significant to cell function as nuclear signaling can lead to altered gene regulation. Shuttling mechanisms are highly regulated [26]. They can be mediated by nuclear localization or export signal (NLS/NES) primary sequences. However, proteins < 40-60 kDa can diffuse through the nuclear pore complex. Diffusion may play a role with GULP1's (approx. 39 kDa) nuclear trafficking, but not with Fe65 (80 kDa). Fe65 has a putative NLS and NES [22-23], which may explain LMB's inhibitory effect on Fe65 exportation from the nucleus. GULP1, however, lacks a "classical" NLS/NES sequence to account for LMB's similar effect on its localization. GULP1 therefore may be transported along with another protein sensitive to LMB treatment in a complex from the cytoplasm to the nucleus. It seems that nuclear shuttling of Fe65 and GULP1 is mediated mechanistically different.

Since overexpression of proteins in cell lines is to some extent artificial, we also examined the nuclear trafficking properties of endogenous GULP1 by immunostaining and a nuclear extraction assay. Endogenous GULP1 could be detected within the nucleus as well, even under control conditions.

It seemed that treatment with LMB had only a minor effect on nuclear localization of endogenous GULP1 which fits our expectations. Surprisingly, LMB treatment caused further accumulation of the overexpressed protein. There might be some impact of inhibiting nuclear export when the signal is amplified due to overexpression and enhanced detection with the epitope tag.

In conclusion, both endogenous and overexpressed GULP1 showed nuclear localization, indicating that nucleocytoplasmic shuttling is also of importance in the physiological system.

### Adaptor proteins Fe65 and GULP1 influence nuclear signaling of AICD or LICD

Fe65 has already been shown to stimulate nuclear transactivation of APP in a γ-secretase dependent manner together with Tip60 [6]. Fe65 mediates transactivation of LRP1 analogously [10, 21]. Therefore, we investigated whether GULP1 might exhibit similar effects.

While our results confirmed previous reports showing that Fe65 enhanced AICD transactivation [6], co-expression of GULP1 did not alter AICD transactivation significantly or promote the effect of Fe65 on APP-Gal4 transactivation, indicating that GULP1 does not affect γ-secretase dependent transactivation of AICD. These findings are in line with our prior finding that GULP1 lowered endogenous production of Aβ40/42 but did not directly influence γ-secretase function [16]. Recently, it was demonstrated that overexpression of GULP1 influences APP–Gal4 dependent transcription by approximately 6–8-fold in transfected CHO, HEK-293 and SH-SY5Y neuroblastoma cells [17]. These different findings could be explained by the use of different APP-Gal4 constructs. We used a APP-Gal4 vector in which the Gal4 was engineered into the intracellular tail of full-length APP695 at the cytoplasmic boundary of the transmembrane region [6]. In contrast Hao*et al.*used a vector consisting of the human APP695 followed by a flexible hinge of ten glycines and by the entire yeast transcription factor Gal4 [17, 27].

As LRP1 also undergoes processing by γ-secretase (releasing LICD), the same experimental setup was chosen to examine possible effects of Fe65 and GULP1 on LICD signaling. Previous studies demonstrated that LICD translocated to the nucleus and interacted with Tip60, but did not function as a transcriptional activator by itself [11]. Furthermore, May *et al.* reported that LICD-mediated transactivation is only enhanced in serum-deprived cells but not in cells grown in serum-containing medium [10]. However, von Arnim*et al.* found that LICD mediates transactivation of a luciferase transporter plasmid and co-transfection of β-site amyloid precursor protein cleaving enzyme 1 (BACE1) led to a significant increase in luciferase activity, suggesting that BACE1 overexpression leads to an increase of γ-secretase-like cleavage to release LICD, which then translocates to the nucleus [21]. Since LC, the 85 kDaβ-chain of LRP1, is responsible for the interaction with adaptor proteins, we decided to analyze transactivation activity of this fragment. Transactivation was observed when LRP1-LC was co-expressed together with Fe65. GULP1, however, has an even more potent effect on LICD transactivation. Both halves of GULP1 are necessary for transactivation, which may indicate that dimerization of GULP1 via the leucine zipper domain is needed for transactivation [28]. LRP1-LC-Gal4 encodes for the additional transcription enhancer VP16 leading to high transactivation activity even without co-transfected adaptors [25]. No additive effect was observed when both Fe65 and GULP1 were co-expressed with LRP1-LC-Gal4, indicating no synergistic effect. Interestingly, co-transfection of Fe65 diminishes transactivation mediated by GULP1, but the observed effect was not significant. Whether GULP1 mediates the interaction of Fe65 and LRP1-LC has to be further investigated.

To further confirm our result that GULP1 mediates LRP1-LC-Gal4 transactivation we performed knockdown experiments. Since endogenous GULP1 levels are very low in Neuro-2A cells and we wanted to confirm our results in a different, human cell line, HEK293 cells were used. Despite their origin, HEK293 cells exhibit some characteristics of neurons. Shaw *et al.* found that HEK293 cells express several proteins typically found in neurons [29]. Another important feature is a high endogenous GULP1 expression. Immunoblotting showed nearly 50% reduced expression of endogenous GULP1. Knockdown reversed the effect of overexpressed GULP1 on LICD transactivation leading to significantly decreased tranactivational activity of LRP1-lc-Gal4. Surprisingly, LICD transactivation was still reduced upon co-transfection of Fe65 suggesting that GULP1 is required for Fe65-mediated transactivation of LICD. The exact mechanism remains unknown and has to be further investigated.

Taken together, our data indicate that LRP1-LC is transcriptionally active depending on co-expression of GULP1.

## Membrane proteins APP and LRP1-LC influence nuclear trafficking of Fe65 and GULP1 differentially

In this study, we compared signaling properties of PTB-domain containing adaptor proteins Fe65 and GULP1. The first step was to compare cellular localization. Since both have been shown to be nucleocytoplasmic shuttling proteins and to influence nuclear signaling properties of AICD and LICD, we investigated the effect of both transmembrane proteins on nuclear trafficking of their adaptors. Confocal images revealed that they co-localized in perinuclear structures and the effect was clearly diminished upon co-expression with NPxY-mutants in both cases. However, a small overlap was still visible.

The effects of APP695 and LRP1-LC on nuclear trafficking of their adaptors differed. APP695 retained both adaptor proteins in the cytoplasm. However, LMB treatment revealed thatboth Fe65 and GULP1 are still released to nuclear trafficking. In contrast, LRP1 retained GULP1 but not Fe65 in the cytosol and did not completely inhibit its nuclear trafficking. Different effects could be due to different interaction sites and other structural elements determining binding. LRP1-LC has two NPxY-interaction sites, but it is not completely clear, which is essential for binding of Fe65. For instance, Fe65 bound to the second motif in pull-down experiments of rat brain lysates incubated with peptides corresponding to the first or second motif [30], the same was reported in a yeast-two-hybrid system [15]. Pietrzik and colleagues showed that Fe65 can bind to both NPxY-motifs, which was also shown by pull-down experiments of overexpressed deletion mutants [4]. However, GULP1 has been reported to bind to the second NPxY-motif [18].

Minopoli*et al.* proposed that Fe65 is anchored in the cytosol by APP [22]. While we also found that APP695 increased Fe65 localization outside the nucleus, LMB treatment revealed that Fe65 can still shuttle between nucleus and cytoplasm, possibly in complex with the AICD [24, 31].

We did not directly detect AICD in the nucleus, which may be due to high perinuclear APP-signals and/or AICD degradation, which has been reported to be rapid and mediated by insulin degrading enzyme[32-33].

## GULP1 transactivation is mediated by LRP1-LC

Since both Fe65 and GULP1 impact APP695 and LRP1-LC transactivation and cycle between cytoplasm and nucleus, the transcriptional capability of the adaptors themselves was tested. Gal4-GULP1 alone or in co-expression with APP695 did not exhibit transactivation. However, Gal4-GULP1 co-expressed with LRP1-LC showed increased

transcriptional activity, indicating that it does actively regulate transcription together with LRP1-LC. These results are in line with the reverse experiment, in which GULP1 does not influence AICD transactivation but does increase LICD transactivation. In contrast to our other reporter gene-based transactivation assays the transcriptional activity of Gal4-GULP1 itself is very low. It could be that GULP1 needs additional stabilization factors for transactivation. However, GULP1 misses a NLS/NES sequence, but it is also sensitive to LMB. GULP1 therefore might be transported along with another protein to the nucleus sensitive to LMB treatment and only functions as co-factor in transcription. This is the first study identifying GULP1 as nucleocytoplasmic shuttling protein, which could be involved in regulation of gene expression. There are no predicted DNA binding sites within the GULP1 sequence, which further promote a role as co-factor. Whether GULP1 directly binds to DNA and can modulate transcription by itself has to be further investigated.

Fe65-Gal4, on the other hand, was able to transactivate the reporter plasmid when co-expressed with APP695 as well as with LRP1-LC. Fe65 has been proposed to have transcription factor properties [34-35], activating a variety of promoters even without APP695-co-expression [36]. GULP1 co-expression increased LRP and APP695 mediated Fe65 transactivation, indicating that GULP1 itself might act as a stabilizer.

Results of the luciferase reporter assays were consistent with observations made in ICC experiments. APP695 retained Fe65 in the cytosol apparently, but did not inhibit nuclear trafficking and signaling as examined with LMB experiments. On the other hand, Fe65 was not held back in the cytosol by LRP1-LC resulting in enhanced transactivation compared to co-expression of APP695.

The same effect was observable for GULP1. Upon APP695 co-expression GULP1 was nearly completely retained in the cytosol and did not transactivate the reporter plasmid, whereas GULP1 could still translocate to the nucleus upon LRP1-LC co-expression resulting in transcriptional activity.

## Conclusions

Our data underline the important role of adaptor proteins in AD and are in line with findings implicating defects in the cell's nuclear trafficking in other neurodegenerative diseases, including RNA-binding protein fused in sarcoma (FUS) in frontotemporal dementia (FTLD) and amylotrophic lateral sclerosis (ALS) [37], altered transcription factor nuclear trafficking in neurodegeneration[38], TDP-43 in ALS/FTLD and FOXO3a in Lewy-Body dementia [39].

This is the first study identifying GULP1 as nucleocytoplasmic shuttling protein mediating transactivation together with LRP1-LC. Since both endogenous and overexpressed GULP1 showed nuclear localization, we addressed the potential physiological role of GULP1 in transactivation by use of fusion proteins encoding the yeasttranscription activatorprotein Gal4. Our data showing that LRP1-LC and GULP1 synergistically transactivate however do not fully explain the nuclear shuttling properties of the adaptor. Therefore, an additional role of GULP1 in the nucleus has to be proposed. The next important step would be the identification of an endogenous DNA binding protein involved in this process.

GULP1 and LRP1 were already shown to interact with each other mediating engulfment of apoptotic cells [18-20]. Our observations raise the possibility that the GULP1-LRP1 interaction has a role beyond enhancing endocytosis or autophagy and suggests that there may also be a transcriptional consequence to GULP1-LRP1 interactions during apoptosis. These new findings contribute to the understanding of the physiological role of GULP1, but also its role in the pathogenesis of AD.

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## Abbreviations used

| | |
|---|---|
| **Aβ** | Amyloid β |
| **AD** | Alzheimer's Disease |
| **AICD** | APP intracellular domain |
| **ALS** | amylotrophic lateral sclerosis |
| **A4** | Amyloid-β |
| **APP** | Precursor Protein |
| **BACE1** | β-site cleaving enzyme 1 |
| **CED** | *C. elegans* death |
| **CLSM** | confocal laser scanning microscopy |
| **FTLD** | frontotemporal dementia |
| **FUS** | fused in sarcoma |
| **GULP1** | engulfment adapter PTB domain containing 1 |
| **HEK293) cells** | human embryonic kidney cells |
| **HA** | hemagglutinin |
| **ICC** | immunocytochemistry |
| **LICD** | LRP1 intracellular domain |
| **LMB** | Leptomycin B |
| **LRP1** | low density lipoprotein receptor-related protein 1 |
| **LRP1-LC** | light chain of LRP1 |
| **NES** | nuclear export signal |
| **NLS** | nuclear localization signal |
| **PTB** | phosphotyrosine binding |
| **RLU** | relative light unit |

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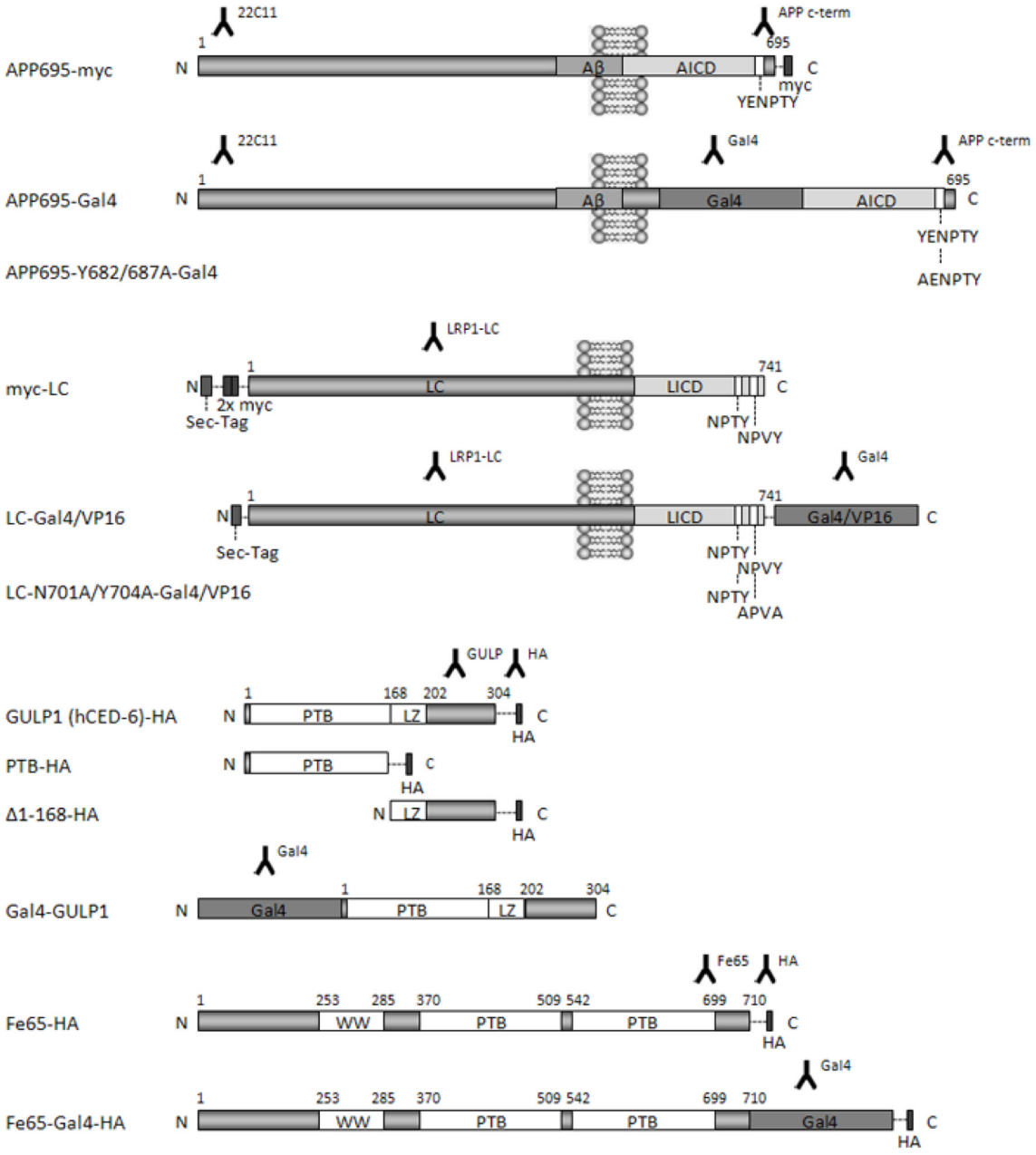


**FIGURE 1. Overview of protein constructs used in this study**

All plasmids used for ICC were tagged with a C-terminal HA- or myc-tag and generated as described in materials and methods section. Antibodies for protein detection are indicated.

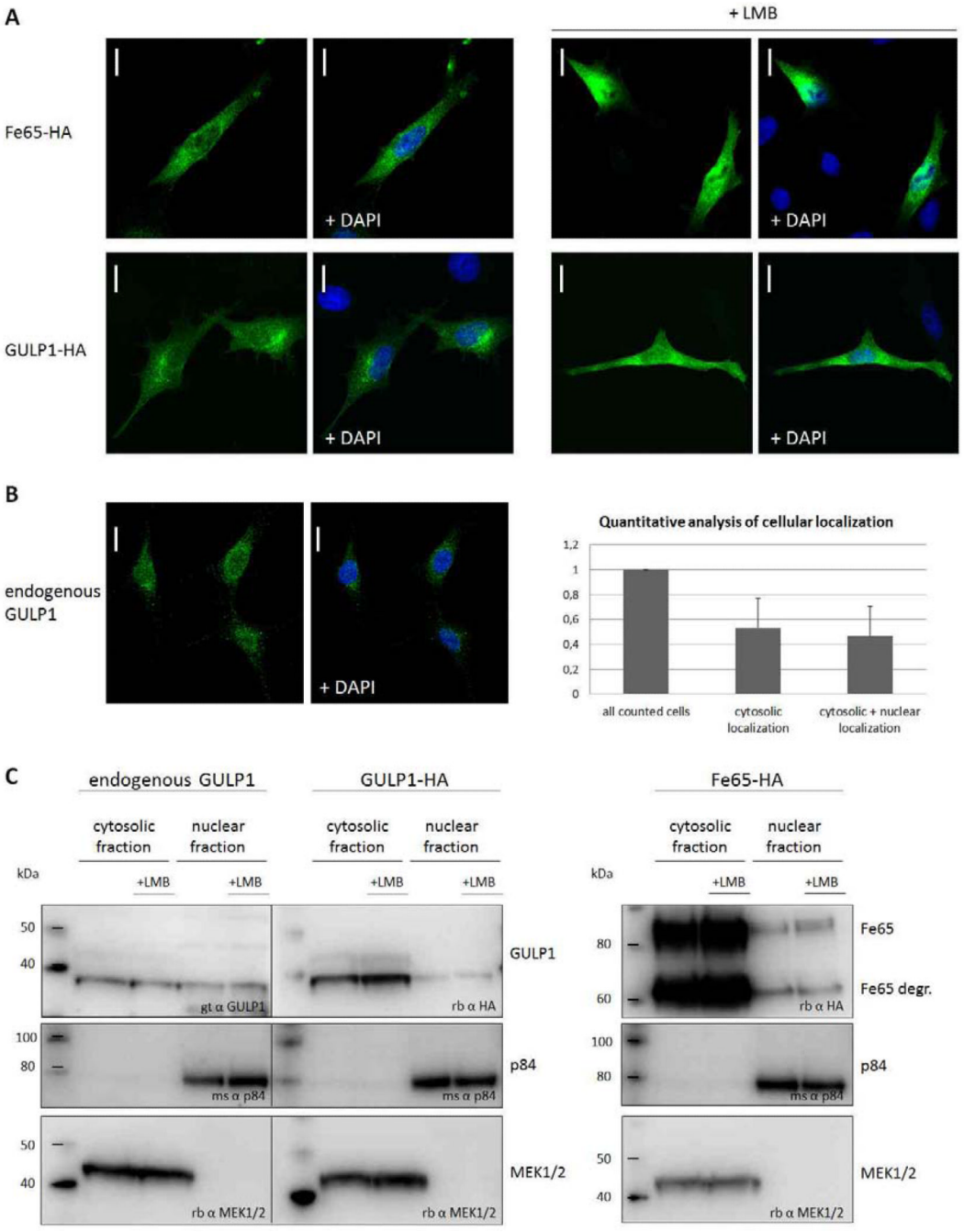


**FIGURE 2. GULP1 and Fe65 are nucleocytoplasmic shuttling proteins**

(A) Neuro-2A cells were transfected with Fe65-HA or GULP1-HA, stained with a tag-directed antibody and fluorescent-labeled secondary antibody to examine their cellular distribution. Fe65 localized both in the cytoplasm and in the nucleus, whereas GULP1 was found mostly in the cytoplasm. However, administration of LMB (5ng/ml, 2h) revealed that GULP1 is also a nucleocytoplasmic shuttling protein. Bar: 10μm (B) To check whether endogenous GULP1 can also be detected within the nucleus, Neuro-2A cells were immunostained with a GULP1 specific antibody. GULP1 was found mainly in perinuclear region but also within the nucleus, even without LMB treatment. Bar: 10μm. Quantitative analysis was carried out showing that nearly 50% of all counted cells were positive for

nuclear localization of GULP1. Data are mean values ± SEM. (C) To investigate whether ICC results are consistent with another approach, a nuclear extraction assay was performed. Equal amounts of nuclear extract or cytosolic fraction were subjected to immunoblotting. Purity of fractions was determined by detection of nuclear (p84) or cytosolic (MEK1/2) marker proteins. Both endogenous and overexpressed GULP1 were found within the nuclear fraction. Fe65 was used as control.

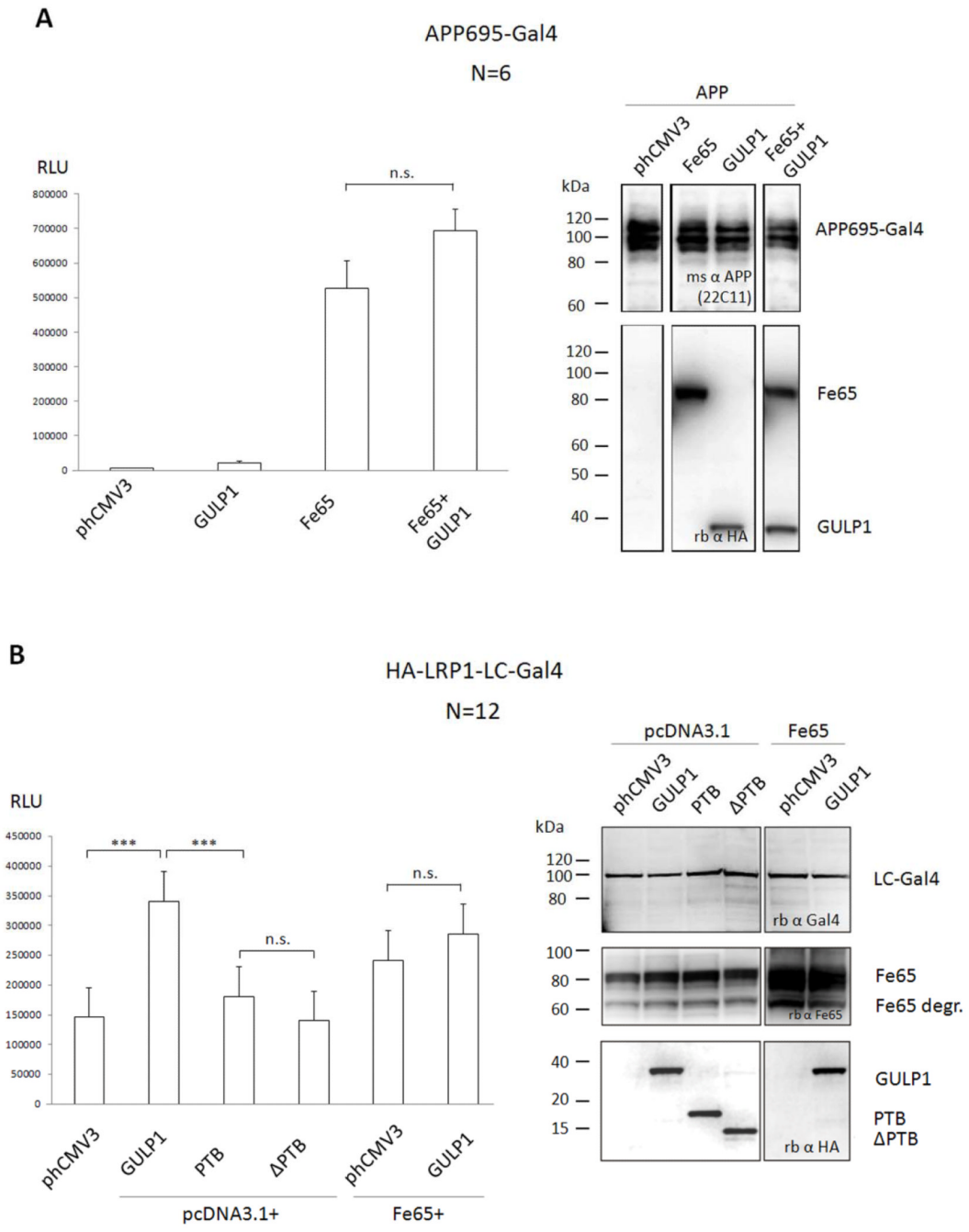


**FIGURE 3. Fe65 mediates transactivation of both AICD and LICD, while GULP1 only enhances LICD transactivation**

Neuro-2A cells were transfected with β-gal, pG5E1B-luc, Gal4-construct, adaptor(s) and/or empty vector. Expression of adaptors was confirmed by immunoblotting. Luciferase was measured in duplicate and normalized to β-gal activity. Our results were consistent with previous experiments and showed that Fe65 triggers APP-Gal4 transactivation [6] to a greater extent than LRP1-LC-Gal4 [11]. GULP1 enhanced LRP1-LC-Gal4 transactivation more than Fe65 (B) and had no effect on APP-Gal4 transactivation (A). N=6 (A) and N=12 (B), 3 independent repetitions, p-value by Mann-Whitney Rank sum test ***<0.001.

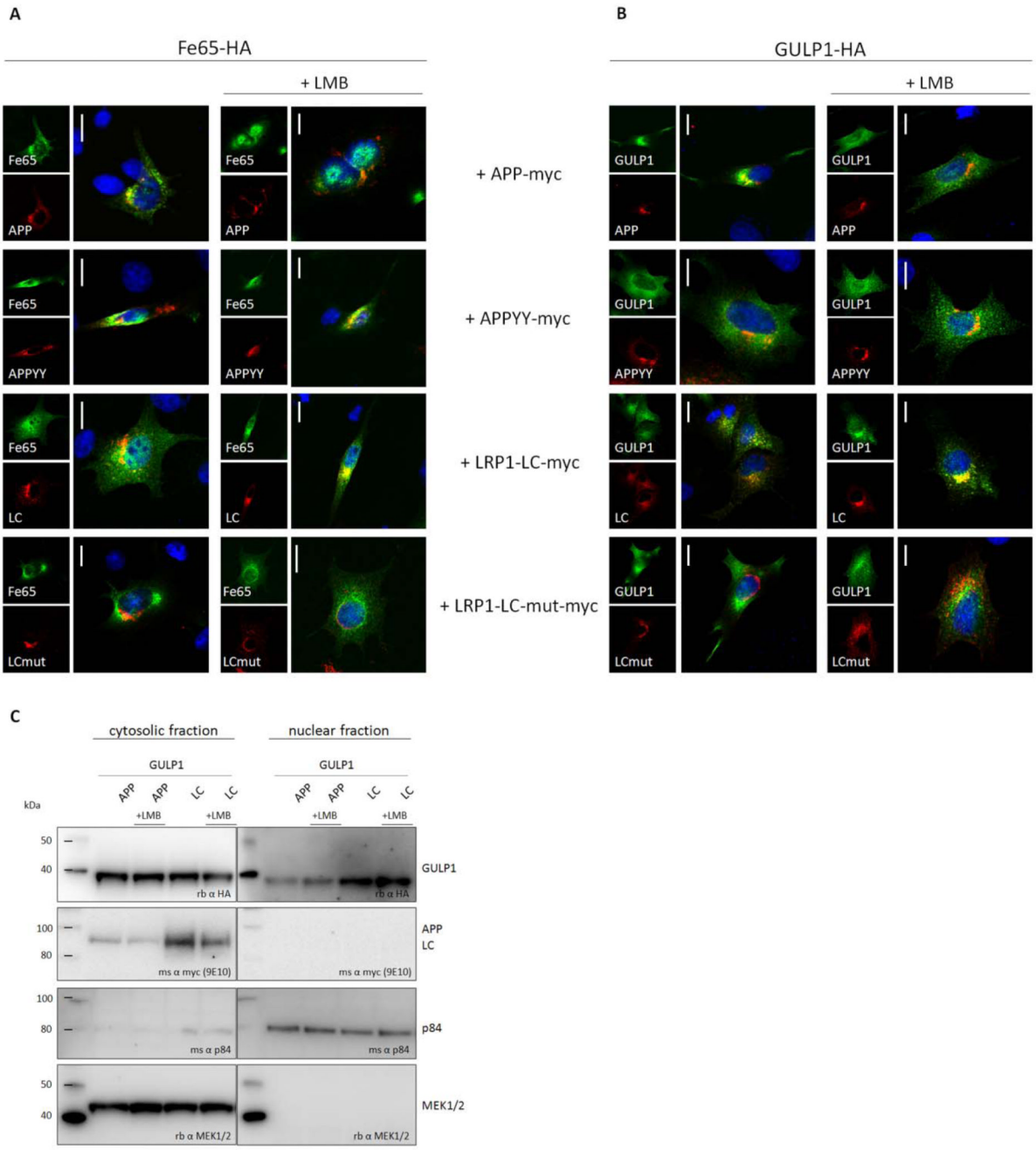


**FIGURE 4. APP or LRP1-LC co-expression retains Fe65 and GULP1 in the cytosol depending on PTB-YENPTY interaction**

Neuro-2A cells were co-transfected with Fe65 or GULP1 and APP695 or LRP1-LC and the cellular distribution was analyzed by CLSM. (A) Co-expression of APP695 caused enrichment of Fe65 in the cytosol. LMB treatment revealed that Fe65 still shuttled between nucleus and cytoplasm. LRP1-LC co-expression did not prevent Fe65 from localizing to the nucleus. (B) GULP1 co-localized with APP695 and was nearly completely retained in the cytosol by APP695. However, upon LMB treatment, GULP1 was enriched in the nucleus. Upon LRP1-LC co-expression, GULP1 co-localized with LRP1-LC. However, LMB treatment revealed that GULP1 was not completely retained in the cytosol. The observed

effects depended on PTB-domain/YENPTY-motif interaction since co-expression of APP-$Y^{682/687}$A-mutant or LRP-LC-$N^{4504}A/Y^{4507}$A-mutant resulted in both adaptors having similar nucleocytoplasmic shuttling patterns as when they were transfected alone. Bar: 10μm

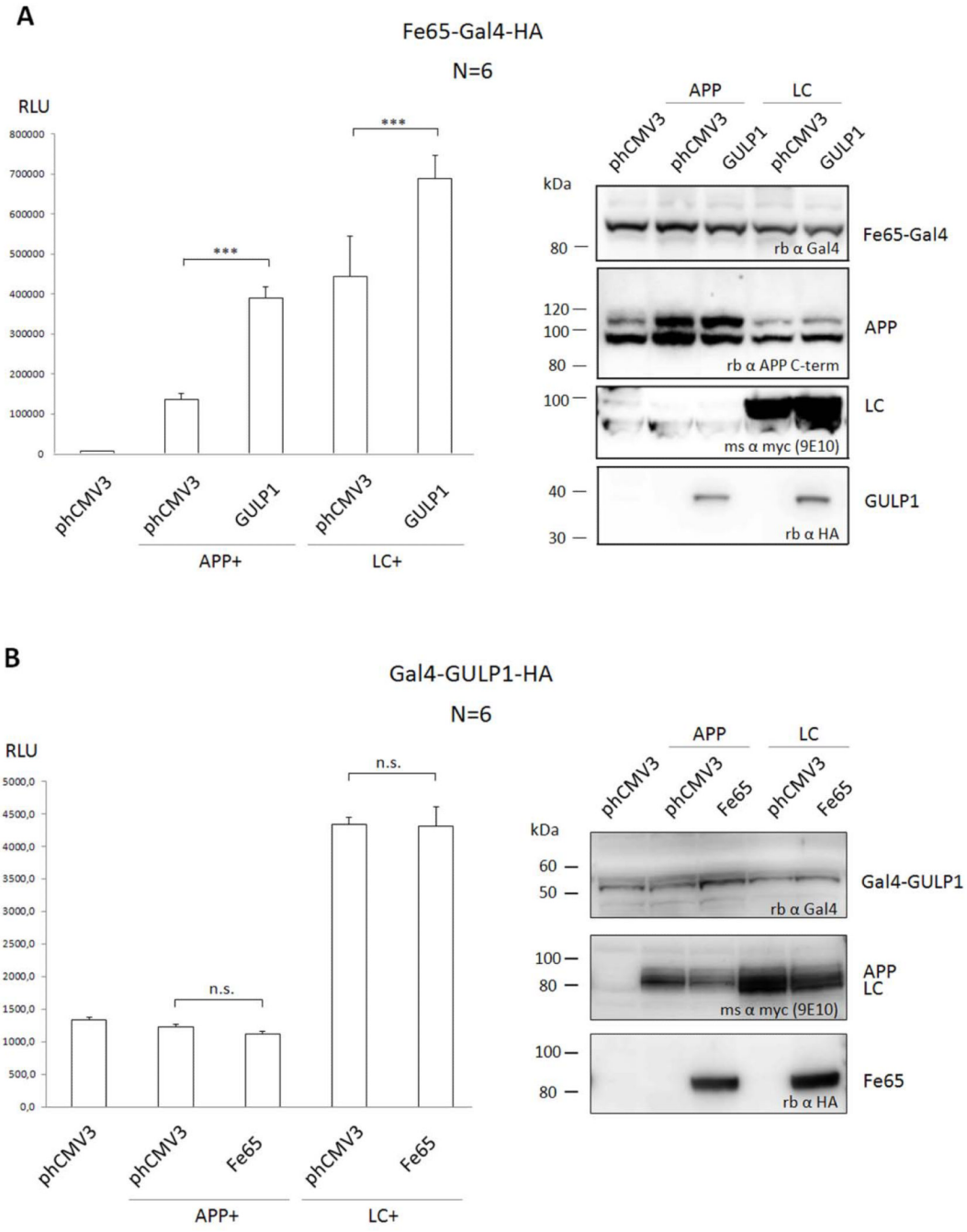


**FIGURE 5. APP and LRP-LC trigger transactivation of Fe65, while GULP1 transactivation is only enhanced by LRP-LC co-expression**

Transcriptional activity of Fe65[6] or GULP1 was tested. Neuro-2A cells were transfected with β-gal, pG5E1B-luc, Gal4-construct, APP695/LRP-LC and/or empty vector and equal expression was confirmed by immunoblotting. Luciferase was measured in duplicate and normalized to β-gal activity. Upon co-expression of APP695 or LRP1-LC, Fe65-Gal4 showed increased transcriptional activity compared to Fe65-Gal4 expression alone. Interestingly, GULP1 co-expression boosted the effect of APP695 and LRP1-LC on Fe65 transactivation (A). Gal4-GULP1 showed no transcriptional activity when transfected alone or in combination with APP695. However, co-expression of LRP1-LC led to increased

transcriptional activity (B). N=6, 3 independent repetitions, p-value by Mann-Whitney Rank sum test ***<0.001.

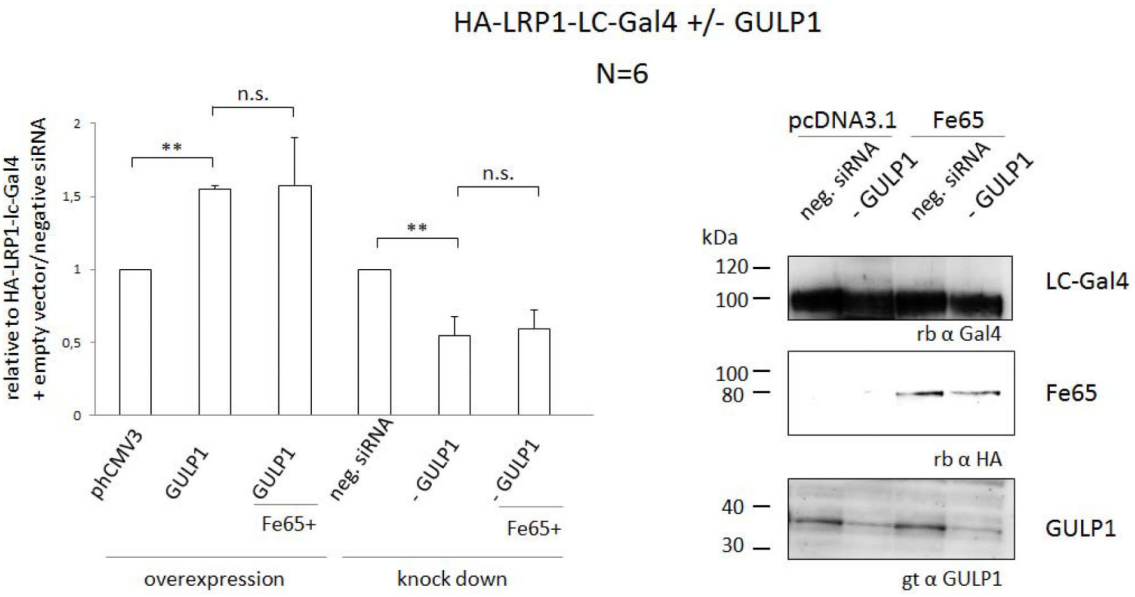


**FIGURE 6. Knockdown of GULP1 inhibits LICD transactivation**

HEK293 cells were transfected with β-gal, pG5E1B-luc, HA-LRP1-lc-Gal4, GULP1 siRNA, Fe65 or empty vector. Expression of adaptors was confirmed by immunoblotting. Luciferase was measured in duplicate and normalized to β-gal activity. Our results showed that the effect of overexpressed GULP1 on LICD transactivation is reversible by knockdown of GULP1 using specific siRNA. N=6, 3 independent repetitions, p-value by Mann-Whitney Rank sum test **<0.01.