

Optimization, Functionalization and Application of Nanobodies against Miro1 and Drp1 to study Mitochondrial Dynamics

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List of publications and personal contributions

1) A Nanobody-Based Toolset to Monitor and Modify the Mitochondrial GTPase Miro1

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2) 30 years of nanobodies - an ongoing success story of small binders in biological research

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This publication was not included in this dissertation.

3) Nanobodies as novel tools to monitor the mitochondrial fission factor Drp1

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Publication	Author position	Scientific ideas %	Data Generation %	Analysis & Interpretation %	Paper Writing %
1)	6	5	10	5	5
2)	2	R	R	R	30
3)	1	50	50	25	70

R: review article

A detailed description of my contributions can be found at the beginning of the respective chapters.

Abbreviations

Ab/Abs	antibody/antibodies
AD	Alzheimer's disease
AF	AlexaFluor
AID	auxin inducible degron
AMCBS	antigen-mediated Cb stabilization
ALS	amyotrophic lateral sclerosis
Arg	arginine
ATP	adenosine triphosphate
BLI	biolayer interferometry
BSA	bovine serum albumin
BSE	bundle signalling element
biv	bivalent
CAR	chimeric antigen receptor
Cb/Cbs	chromobody/chromobodies
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CDR	complementary-determining region
CPP	cell penetrating peptide
cho-coo	cholesterol-coomassie
CI	confidence interval
CV	column volume
DAPI	4,6-diamidino-2-phenylindole
DBCO	dibenzocyclooctyne
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DNA-PAINT	DNA-based point accumulation for imaging in nanoscale topography
Dox	Doxycycline
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
EMA	European medicines agency
ER	endoplasmic reticulum
FBS	fetal bovine serum
FDA	food and drug administration
FDR	false discovery rate
FP/FPs	fluorescent protein(s)
FR	framework region
FRET	Förster resonance energy transfer
Cryo-EM	cryogenic electron microscopy
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GBP	GFP binding protein
GED	GTPase effector domain
GFP	green fluorescent protein
GPCRs	G-protein-coupled receptors
GTP	guanosine triphosphate
HCAbs	heavy-chain only antibodies
IC-IP	intracellular immunoprecipitation
IF	immunofluorescence

IgG	immunoglobulin G
IP	immunoprecipitation
iPSCs	induced pluripotent stem cells
IPTG	isopropyl β -D-1-thiogalactopyranoside
IR	ischemia-reperfusion
IMAC	immobilized metal affinity chromatography
K _D	dissociation constant
kDa	kilo Dalton
KO	knockout
Lys	lysine
MCS	multiple cloning site
MD	middle domain
mdivi-1	mitochondrial division inhibitor 1
MIM	mitochondrial inner membrane
MOM	mitochondrial outer membrane
MOMP	mitochondrial outer membrane permeabilization
mRNA	messenger ribonucleic acid
MS	mass spectrometry
mtDNA	mitochondrial DNA
MT green	MitoTracker green
Nb/Nbs	Nanobody/Nanobodies
NC	negative control
NHS	N-hydroxysuccinimide
NLS	nuclear localization signal
n.s.	not significant
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	positive control
PCA	principal component analysis
PCR	polymerase chain reaction
PD	Parkinson's disease
PFA	paraformaldehyde
PGs	protein groups
PINK1	PTEN-induced putative kinase protein 1
PMSF	phenylmethanesulfonyl fluoride
POI	protein of interest
PTMs	posttranslational modifications
RING	really interesting new gene
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	room temperature
scFv	single-chain variable fragment
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
Ser	serine
STED	stimulated emission depletion microscopy
STORM	stochastic optical reconstruction microscopy
SPOP	speckle-type poxvirus and zinc finger domain protein
SRM	super-resolution microscopy

SUMO	small ubiquitin-like modifier
TB	terrific broth
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.05% Tween 20
TEMED	tetramethylenediamine
tRNA	transfer RNA
Ub	Ubiquitin
VD	variable domain
VHH	variable domain of HCAs
v/v	volume per volume
wt	wild type
w/v	weight per volume

Abstract

To fulfil their multiple cellular functions, mitochondria constantly adapt to changing requirements by fusion, fission and active transport within the cell. These processes are coordinated by two key proteins: Drp1, which is responsible for the membrane constriction during mitochondrial fission, and Miro1, which connects the mitochondria to motor proteins, thus enabling mitochondrial movement.

Drp1 dysregulation was linked to multiple diseases including Parkinson's and cardiovascular diseases thus making Drp1 an attractive biomarker and a potential therapeutic target. To leverage this potential, a deeper understanding of Drp1 and Miro1 is required, which is currently impeded by the available research tools. To facilitate biochemical and cell biological studies of endogenous Drp1, Drp1-specific nanobodies (Nbs) previously identified by a phage display screen were formatted into versatile research tools. Functionalized into capture matrices, the Drp1-Nbs allowed the enrichment of endogenous Drp1 and thereby the identification of Drp1 interaction partners by proteomic analysis. Furthermore, a fluorophore-labelled bivalent Drp1-Nb (bivD7_{AF647}) was generated and successfully applied for super-resolution STORM microscopy of endogenous Drp1. For real-time imaging of Drp1, Nb-fluorescent protein fusions, so-called chromobodies (Cbs), were intracellularly expressed in mammalian cells and the signal-to-noise ratio further improved by generating "turnover-accelerated" Cbs. With these probes, the dynamic relocalization of endogenous Drp1 upon compound-mediated induction of fission was visualized in living cells. Additionally, the intracellularly functional Drp1-Nbs were engineered into inducible TRIM21-based degrons for targeted depletion of Drp1 in live cells.

For proteomic studies of endogenous Miro1, a previously generated Miro1-specific Nb was formatted into a bivalent affinity matrix, which was successfully applied to analyse the Miro1 interactome.

To establish an approach for bringing fluorescently labelled Nbs into live cells, a recently described cholesterol-coomassie based approach for transducing proteins in live cells was tested for different Nbs. Although successful transduction of the Nbs into the cytosol was observed in principle, the method suffered from high cellular toxicity and low efficiency.

Due to their versatility, the Nb-based research tools developed and validated in this study can facilitate further investigations into Drp1 and Miro1 and thus help answer open questions regarding their role in mitochondrial dynamics and pathophysiology.

Zusammenfassung

Zur Erfüllung ihrer vielfältigen Funktionen passen sich Mitochondrien durch Fusion, Teilung, und aktiven intrazellulären Transport kontinuierlich an unterschiedliche zelluläre Anforderungen an. Zwei zentrale Proteine sind hierbei Drp1, verantwortlich für die Konstriktion der mitochondrialen Membran während der Teilung, und Miro1, welches Mitochondrien an zelluläre Motorproteine koppelt und so ihre Bewegung innerhalb der Zelle ermöglicht.

Mutationen bzw. die Fehlregulation dieser beiden Komponenten sind in verschiedensten Krankheiten wie z.B. Parkinson, kardiovaskulären Erkrankungen und Krebs beschrieben. Dies macht sie zu attraktiven Biomarkern und potenziellen therapeutischen Angriffspunkten. Um dieses Potenzial zu nutzen, bedarf es jedoch eines tiefergehenden Verständnisses der von Drp1 und Miro1 gesteuerten Prozesse, was aufgrund unzureichender Forschungsreagenzien aktuell limitiert ist. Um biochemische und zellbiologische Untersuchungen von Drp1 zu erleichtern, wurden in dieser Arbeit zuvor mittels Phagen-Display-Screens identifizierte Drp1-spezifische Nanobodies (Nbs) zu vielseitigen Forschungswerkzeugen formatiert. Im Affinitätsmatrix-Format ermöglichten die Drp1-Nbs die Anreicherung von endogenem Drp1 sowie die Identifikation von Interaktionspartnern durch Proteomanalyse. Ein fluorophor-markierter bivalenter Drp1-Nb (bivD7_{AF647}) wurde zudem erfolgreich für die Visualisierung von endogenem Drp1 mittels hochauflösender STORM-Mikroskopie verwendet. Für die Echtzeit-Mikroskopie wurden Fusionen der intrazellulär funktionalen Drp1-Nbs mit fluoreszierenden Proteinen, sogenannte Chromobodies (Cbs) in Säugerzellen exprimiert und für eine verbesserte Bildgebung zu „Proteinumsatz-beschleunigten“ Cb-Versionen weiterentwickelt. Diese intrazellulären Sonden ermöglichten die Visualisierung der Wirkstoff-induzierten dynamischen Relokalisation von Drp1 zu Mitochondrien in lebenden Zellen. Intrazelluläre Drp1-Nbs wurden darüber hinaus zu induzierbaren TRIM21-basierten Degrons modifiziert, um Drp1 in lebenden Zellen gezielt abzubauen.

Für Proteomuntersuchungen von Miro1 wurde ein zuvor generierter Miro1-spezifischer Nb in eine bivalente Affinitätsmatrix formatiert und erfolgreich für die Analyse des Miro1 Interaktoms verwendet.

Zusätzlich sollte eine Methode für die Einbringung von fluoreszenzmarkierten Nbs in lebende Zellen etabliert werden. Hierfür wurde ein zuvor publizierter auf Cholesterin-Coomassie basierender Ansatz für die Proteintransduktion für verschiedene Nbs

getestet. Obwohl eine erfolgreiche Transduktion der Nbs ins Zytosol prinzipiell beobachtet werden konnte, zeigte die Methode eine hohe Zelltoxizität sowie eine geringe Effizienz.

Aufgrund ihrer Vielseitigkeit können die in dieser Studie entwickelten und validierten Nb-basierten Forschungswerkzeuge zukünftige Untersuchungen zu den beiden Schlüsselfaktoren Drp1 und Miro1 erleichtern und damit dazu beitragen, offene Fragen zu ihrer Rolle in der mitochondrialen Dynamik und der Pathophysiologie zu beantworten.

1 Introduction

1.1 Mitochondria

When first learning cell biology, mitochondria are introduced as the “powerhouse of the cell” due to their role in energy production¹. However, this term negates the plethora of functions that mitochondria perform. Thought to be derived from endosymbiotic alpha-proteobacteria²⁻⁴, mitochondria consist of four compartments: mitochondrial inner membrane (MIM) and mitochondrial outer membrane (MOM), intermembrane space and mitochondrial matrix⁵. The MIM is tightly folded into cristae hereby increasing the membrane surface^{5,6} and differs in lipid composition from the MOM⁷. Mitochondria form an intricate dynamic network⁸ which is in contact with other organelles like the endoplasmic reticulum (ER)⁹⁻¹¹ and peroxisomes^{9,12}.

Besides the nucleus and chloroplasts in plants, mitochondria are the only organelle containing DNA¹³. Mitochondrial DNA (mtDNA) encodes for 13 proteins of the oxidative phosphorylation machinery as well as 22 tRNAs and 2 rRNAs, while the majority of proteins is nuclear encoded thus requiring import¹⁴.

In addition to their role in cellular energy production via the electron transport chain and ATP synthase⁵, mitochondria participate in lipid¹⁵⁻¹⁷ and amino acid metabolism^{18,19}, steroidogenesis²⁰ and iron-sulfur cluster synthesis²¹⁻²³. Furthermore, mitochondria are involved in Ca²⁺ homeostasis^{24,25} and apoptosis²⁶⁻²⁸.

The importance and versatility of mitochondria for proper cell function is reflected in the multiple and diverse clinical manifestations of mitochondrial diseases including hepatic failure, pancreatitis and respiratory failure as well as neurological symptoms like epilepsy and optic atrophy^{29,30}.

1.2 Mitochondrial dynamics

As mitochondria cannot be formed *de novo*^{31,32}, cells need to continuously maintain a pool of healthy mitochondria. For this, mitochondria constantly fuse and divide to ensure a proper distribution during mitosis^{8,33} and, for example, to remove damaged mitochondria³⁴. In addition, to fulfil their various functions, they must constantly and rapidly adapt to changing cellular conditions.

By mitochondrial fusion, components like mtDNA and metabolites are exchanged, which counteracts the accumulation of mutations and improves respiratory function³⁵⁻³⁷. Hereby, first MOMs and then MIMs fuse in distinct but coordinated processes^{38,39}. In mammals, MOM fusion is mediated by Mitofusin 1 and 2 (Mfn1 and Mfn2) while the key factor MIM fusion is Optic Atrophy-1 (OPA1)³⁹ (**Figure 1**).

Mitochondrial fission enables, among others, the removal of damaged mitochondria by selective autophagy (mitophagy)^{35,40} and is executed by dynamin-1-like protein (Drp1) and its adaptors (**Figure 1**, see section 1.3).

In addition, mitochondria are positioned in subcellular regions with high demand for their functions by active transport along the cytoskeleton⁴¹⁻⁴³. This is especially important in neurons due to their high energy consumption, long lifetime and elongated cell shape⁴⁴. Mitochondria are transported both anterograde and retrograde along microtubules by the motor proteins kinesin-1 and dynein, respectively^{41,42,45}, while short range transport is mediated by actin filaments and Myosin XIX⁴⁶. Mitochondria are connected to the different motor proteins by the adaptors Mitochondrial Rho GTPase 1 and 2 (Miro1 and Miro2) as well as Trafficking kinesin-binding protein 1 and 2 (TRAK1 and TRAK2)^{46,47} (**Figure 1**, see section 1.4).

Together, mitochondrial fission, fusion and movement are summarised under the term mitochondrial dynamics and are crucial for the proper function of mitochondria.

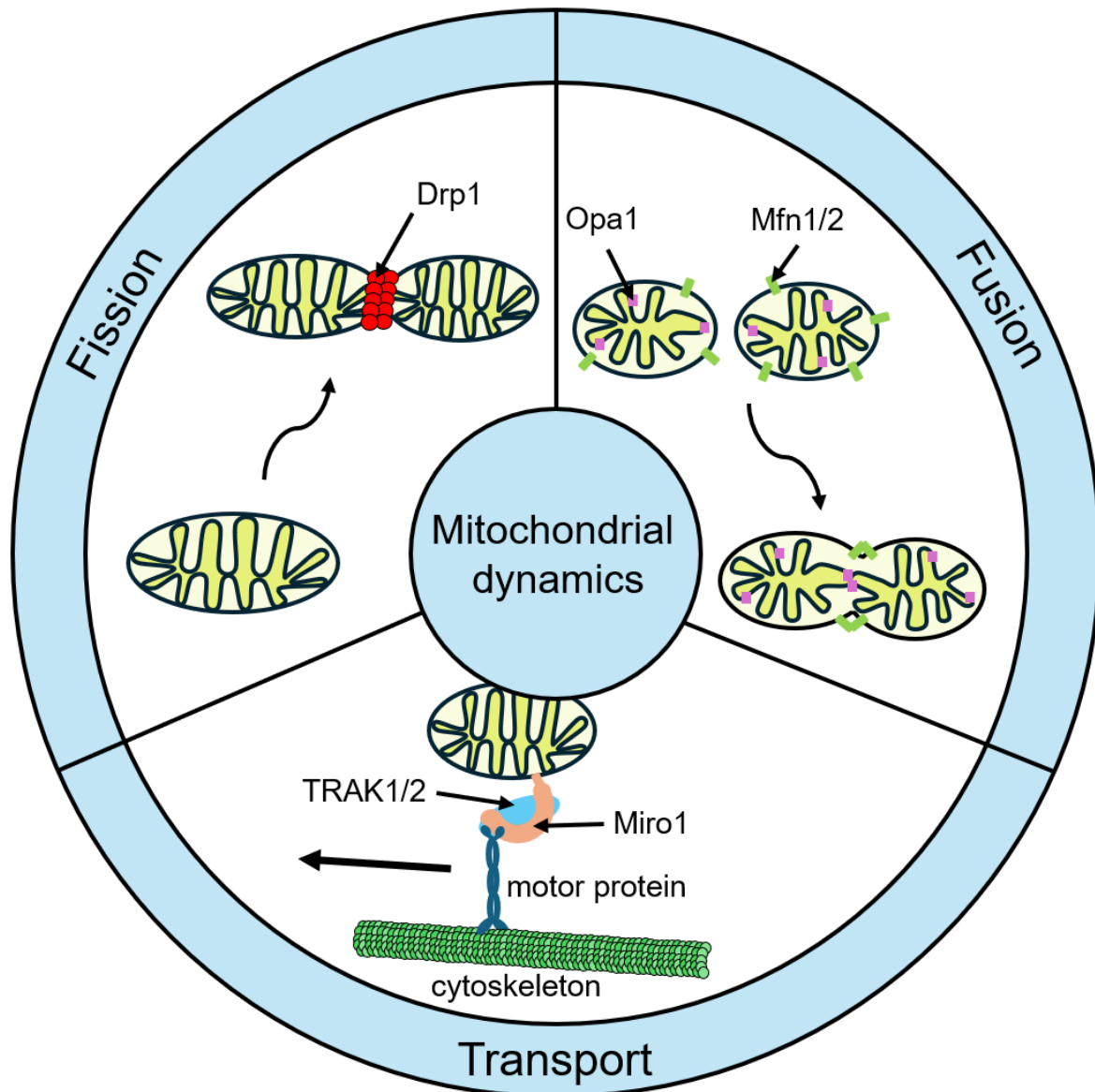


Figure 1: Mitochondrial dynamics

The term “mitochondrial dynamics” summarizes mitochondrial fission, fusion and transport. Mitochondrial fusion of the mitochondrial outer membranes is mediated by Mitofusin 1 and 2 (Mfn1/2) while the mitochondrial inner membranes are fused by Optic Atrophy-1 (OPA1). The opposing mitochondrial fission is mediated by Dynamin-1-like protein (Drp1). In addition, mitochondria are transported along microtubules and actin filaments by different motor proteins, including dynein and kinesin. Hereby, the adaptors Mitochondrial Rho GTPase 1 (Miro1) and trafficking kinesin-binding protein 1 and 2 (TRAK1/2) mediate binding and possess regulatory functions.

1.3 Drp1

Dynamin-1-like protein (*DNM1L*; Drp1 also known as DLP1), a large GTPase of ~80 kDa, is responsible for the membrane constriction during both mitochondrial and peroxisomal fission^{48,49}. As a key player in these processes, Drp1 function is highly regulated by distinct expression patterns of its nine isoforms in different tissues, posttranslational modifications (PTMs) and modulation of the interaction with its receptors^{50,51}. The importance of Drp1 for cellular health is underscored by the lethality of Drp1 KO in mice⁵² and its involvement in several human diseases⁵³⁻⁵⁹.

Drp1 structure

Drp1 consists of four domains: the GTPase domain conferring enzymatic activity, middle domain (MD), variable domain (VD) and GTPase effector domain (GED)^{49,60-62} (**Figure 2**). MD and GED form the stalk which mediates the oligomerization of Drp1^{60,63}. Instead of the pleckstrin homology domain found in dynamins, Drp1 contains the VD^{60,61} which interacts with cardiolipin^{64,65}. The VD modulates Drp1 function by auto-inhibition of Drp1 self-assembly⁶⁴ and contains multiple sites for PTMs⁶¹. In addition, Drp1 contains a bundle signalling element (BSE) which is located across the protein and forms a three-helix bundle (**Figure 2A**). The BSE is responsible for the powerstroke movement leading to membrane constriction^{60,66}.

Drp1 has nine isoforms (nomenclature according to Rosdah *et al.*⁵⁰) encoded by 21 exons which mainly differ by the presence or absence of two sequences: the A- and B-insert⁵⁰. The A-insert (encoded by exon 3) is located in a loop of the N-terminal GTPase domain and promotes Drp1 self-assembly by enhancing the oligomerization propensity⁶⁷. The B-insert (exon 16 and/or 17) located in the VD seems to regulate membrane curvature while both inserts influence the GTPase activity⁶⁷. The isoform 7, on the other hand, might not bear GTPase activity at all due to N-terminal truncations⁶⁸. The expression of Drp1 isoforms varies between cell types and tissues. Isoform 3, which contains neither A-insert nor B-insert, is ubiquitously expressed^{67,69}. Isoforms containing the A-insert (isoforms 6, 8, and 9) as well as isoform 1 (no A-insert but the full B-insert) are mainly expressed in neurons^{50,67,70,71}. Isoform 2 shows high expression levels in spleen, thymus and peripheral blood mononuclear cells (PBMCs) indicating a specialized function in the immune system⁷⁰. As the isoforms differ, for example, in their GTPase activity⁶⁷ and subcellular localization⁷⁰, this differential

expression regulates Drp1 function and can explain conflicting results between studies⁵⁰.

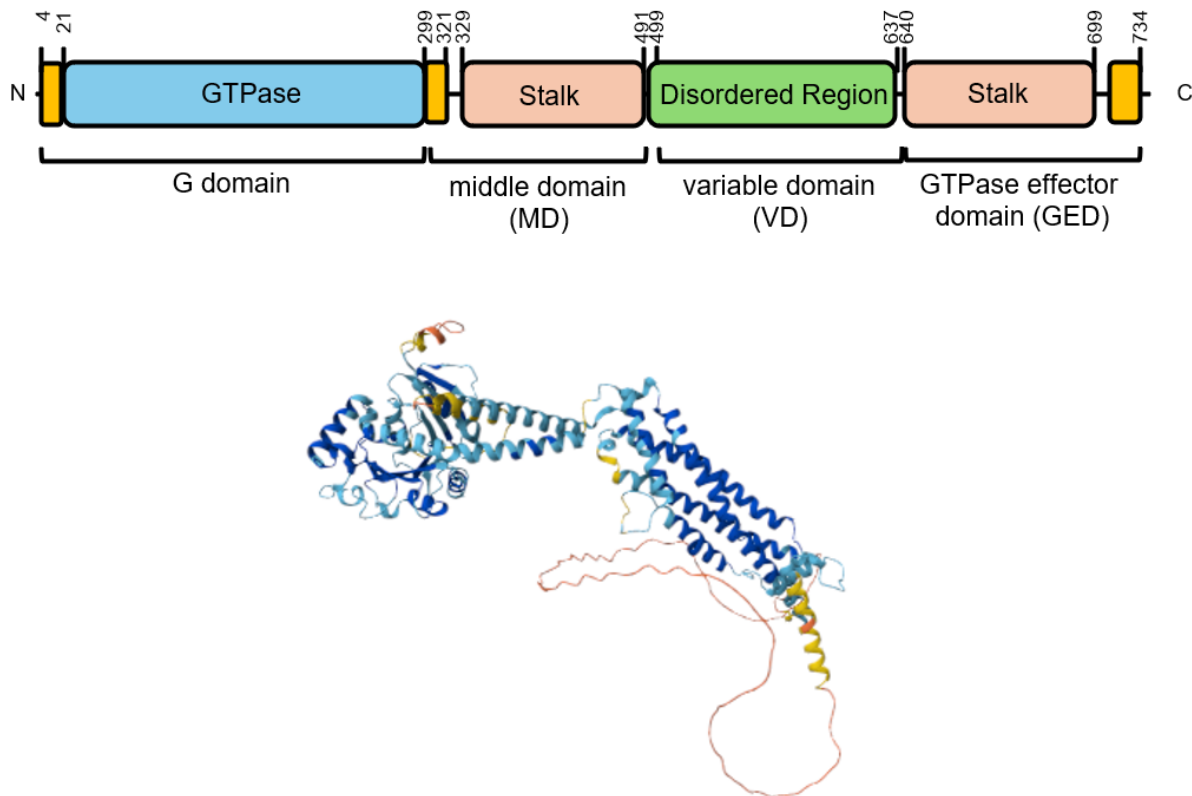


Figure 2: Drp1 structure

A Schematic representation of Drp1 domains and structural elements with bundling signalling element (BSE; yellow), GTPase (light blue), stalk (light brown) and disordered region (green). Domain annotations according to Rochon et al.⁶⁸. **B** AlphaFold prediction of Drp1 isoform1 coloured according to model confidence (dark blue: very high (pLDDT>90), light blue: high (pLDDT >70), yellow: low (pLDDT >50), orange: very low (pLDDT<50)). Structure is taken from the AlphaFold Protein Structure Database with a model creation date of 1 Aug 2025^{72,73}.

Mediation of fission by Drp1

Mitochondrial fission is a multistep process that is initiated, for example, during mitosis⁷⁴. Cytosolic Drp1 is recruited to pre-constricted endoplasmic reticulum (ER)-mitochondrial contact sites⁷⁵ facilitated by inverted formin 2 (IFN2)^{76,77} and actin filaments^{76,78-80}. On the MOM, Drp1 then associates with its receptors: mitochondrial

fission factor (Mff), mitochondrial dynamics protein of 49 kDa (MiD49, also known as MIEF2), mitochondrial dynamics protein of 51 kDa (MiD51; also known as MIEF1), and fission protein 1 (Fis1) which allows Drp1 to oligomerize into ring-like or helical structures around the mitochondrion⁸¹⁻⁸³. The GTP hydrolysis by the Drp1 GTPase activity then results in a conformational change in the BSE^{60,63,66,75,77,84,85} which constricts the MOM^{60,63,66,75,77,84,85} and leads to dissociation between Drp1 and its receptors^{86,87}. While a role of dynamin 2 in the final step of fission was suggested⁸⁸, other studies concluded that Drp1 is sufficient to sever the mitochondrion^{89,90} which finalizes fission (**Figure 3**).

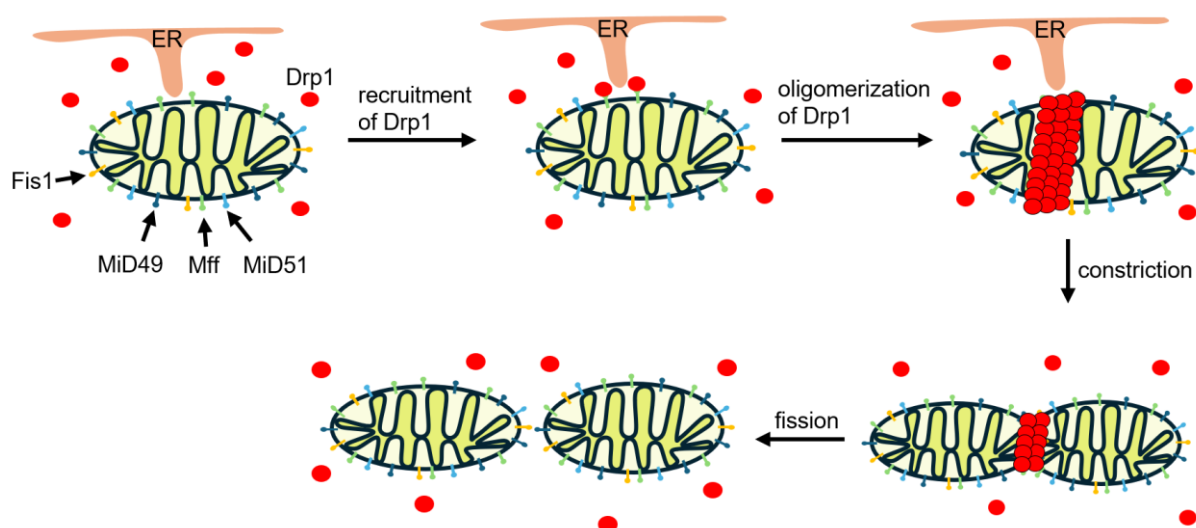


Figure 3: Schematic depiction of Drp1-mediated mitochondrial fission

At ER-contact sites, Drp1 is recruited from the cytosol to the MOM where it interacts with its mitochondrial-anchored adaptors (Mff, MiD49, MiD51 and Fis1) and oligomerizes. Drp1 then constricts the mitochondrion using its GTPase activity leading ultimately to fission.

Drp1 regulation

Drp1 as the central player in mitochondrial fission is highly regulated by a variety of PTMs and interacting partners with the best-studied regulatory modifications being phosphorylation at Ser616 and Ser637.

Phosphorylation at Ser616 increases fission^{33,74,91}, while Ser637 phosphorylation is inhibitory^{92,93} and dominant over Ser616⁹². These phosphorylation sites are addressed by a variety of kinases and phosphatases. For example, PTEN-induced putative kinase

1 (PINK1), a key player of mitochondrial quality control and in early-onset Parkinson's disease (PD)⁹⁴, phosphorylates Drp1 at Ser616 to induce fission prior to mitophagy^{54,95}. In addition, Ser616 is phosphorylated by Cdk1/cyclinB during mitosis⁷⁴. Ser637, whose phosphorylation also follows a circadian rhythm⁹⁶, is phosphorylated by cAMP-dependent protein kinase (PKA)^{93,97} and dephosphorylated by calcineurin which modulates apoptosis⁹⁷.

Drp1 was also found to be SUMOylated by all three small ubiquitin like modifier (SUMO) isoforms with different degrees of SUMOylation depending on the Drp1 isoform⁹⁸⁻¹⁰⁰. In reperfusion after ischaemia, for instance, SENP3 deSUMOylates Drp1 resulting in increased Drp1 localisation to the MOM which promotes cell death^{101,102}.

While the existence of four Drp1 receptors seems redundant at first glance, it appears that they function in different contexts of mitochondrial fission. For instance, Mff is considered the main adaptor at steady-state as Mff-knockout (Mff-KO) cells show the strongest morphological changes of Drp1 receptor KOs^{81,103,104}. Fis1 seems to have a more specialized role in mitophagy^{81,105-107} while the MiDs are involved in apoptotic mitochondrial fission (see below)¹⁰⁴.

Drp1 and cell death

Besides maintaining mitochondrial dynamics in equilibrium, Drp1-mediated fission is also involved in apoptosis¹⁰⁸. In intrinsic apoptosis, Bax or Bak, pro-apoptotic proteins of the BCL-2 family, are activated by an apoptotic stimulus, e.g. irreparable DNA damage, and accumulate on the mitochondria where they form pores resulting in mitochondrial outer membrane permeabilization (MOMP). Cytochrome c is hereby released into the cytosol leading to caspase activation and ultimately cell death¹⁰⁹.

Several studies have demonstrated Drp1 interaction and/or colocalization with Bax^{108,110,111}, which is suggested to stabilize Drp1 on mitochondria¹⁰⁰. This stabilization can be further increased though Drp1 SUMOylation by mitochondrial-anchored protein ligase (MAPL)^{99,100}. Interestingly, Mff-KO only minorly affected cytochrome c release while MiD49- and MiD51-KO or MiD49+MiD51-KO resulted in strong impairment, a phenotype that is also observed in Drp1-KO cells¹⁰⁴. Thus, MiD49 and MiD51 seem to be the main Drp1-receptors for apoptotic fission.

In addition to its role in apoptosis, Drp1 was also linked to two other forms of cell death: ferroptosis¹¹² and necroptosis¹¹³.

The role of Drp1 in peroxisomes

Drp1 not only mediates the fission of mitochondria, but also that of peroxisomes⁴⁸. Peroxisomes are membrane-bound organelles important for the lipid metabolism including β -oxidation as well as reactive oxygen species (ROS) management¹¹⁴⁻¹¹⁷.

As in mitochondria, Drp1 mediates peroxisomal fission in concert with its adaptors Mff and Fis1, which are localized to both mitochondria and peroxisomes¹¹⁸. At peroxisomes, all three can interact with the peroxisomal protein Pex11¹¹⁸⁻¹²⁰, which mediates the peroxisomal elongation prior to fission^{121,122}, and its overexpression leads to increased peroxisomal proliferation^{48,123,124}. Expression of the dominant negative Drp1 K38A mutant completely inhibited peroxisomal fission⁴⁸ highlighting the central role of Drp1 in this process.

Drp1 and disease

Dysregulation of Drp1 was found in neurodegenerative diseases like Alzheimer's^{53,59}, Huntington's^{57,58} or Parkinson's⁵⁴ as well as cardiovascular diseases^{55,56,125} and cancer^{91,126-129}. Consequently, Drp1 has gained increasing attention both for its pathophysiological role and as a potential therapeutic target.

In hippocampal tissue of Alzheimer's disease (AD) patients, Drp1 levels were found to be reduced while the activating Ser616 phosphorylation was increased compared to an age-matched control group⁵⁹. Treatment of two AD mouse models with mitochondrial division inhibitor 1 (mdivi-1) decreased amyloid deposition and cognitive deficits^{130,131}. Mdivi-1 is a small molecule that was initially described as an Drp1 inhibitor¹³². Since then, it has been widely used for studying Drp1 function and was tested as a potential treatment for Drp1-related diseases^{128,130,131,133-138}. However, in recent years, its Drp1 specificity has been questioned¹³⁹⁻¹⁴¹.

Huntington's disease, an incurable hereditary neurodegenerative disease caused by expansion of the glutamine-coding CAG-repeats in the huntingtin gene, is leading to progressive cognitive and motor impairments¹⁴². Drp1 was found interacting with huntingtin aggregates resulting in increased Drp1 GTPase activity and altered mitochondrial dynamics^{57,58}. Like in AD, increased levels of Ser616 phosphorylation as well as S-nitrosylation were found in Huntington's disease mouse models^{143,144}.

In addition, Drp1 dysregulation has been described in PD. PD is the second most common neurodegenerative disease characterized by loss of dopaminergic neurons

in the substantia nigra resulting in a combination of motor and non-motor symptoms generally requiring a multimodal treatment regimen^{145,146}. Lower Drp1^{Ser616}-phosphorylation levels were identified in patient-derived fibroblasts from four PD patients carrying *PINK1* mutations and four out of seven patients with sporadic PD compared to healthy controls⁵⁴. Moreover, Drp1 is an interaction partner of LRRK2^{147,148}, a protein linked to both sporadic and familial PD¹⁴⁹. In this context, cells expressing the PD-associated LRRK2 mutations R1441C and G2019S showed increased levels of mitochondrial Drp1 compared to wildtype LRRK2¹⁴⁸.

Besides neurodegenerative diseases, increased Drp1 expression and pro-fission associated phosphorylation at Ser616 were detected by both immunoblotting and immunocytochemistry in different types of cancer, including glioblastoma, melanoma, lung, thyroid, breast, pancreatic as well as head and neck cancer^{54,91,126-129,150,151}. It was theorized that the increased Drp1 activity promotes higher mitochondrial fragmentation and thus enhanced aerobic glycolysis which promotes the growth and metastasis of tumour cells^{54,127}. This is in line with findings describing that downregulation of Drp1 resulted in reduced cancer cell proliferation and increased spontaneous apoptosis^{91,127,128,152}.

Increased Drp1-mediated fission was also found in multiple acute cardiovascular diseases like myocardial infarction¹⁵³, cardiac arrest¹⁵⁴ and ischemia¹²⁵. Drp1-inhibition by mdivi-1 reduced ischemia-reperfusion (IR) injury in cell culture heart tissue^{125,155,156}. Administration of P110, which disrupts the Drp1-Fis1 interaction, at the onset of reperfusion reduced damage two hours after IR by 30 % and showed improved cardiac function three weeks post IR in an animal model¹⁵⁷. While long-term blockage of Drp1 is considered harmful^{55,56}, short-term Drp1 inhibition could be an effective treatment to reduce tissue damage by overshooting mitochondrial fission in acute cardiovascular disease.

Cardiovascular and neurodegenerative diseases as well as cancer are major healthcare challenges which will gain even more importance with aging populations¹⁵⁸⁻¹⁶⁰. Thus, Drp1 modulation has considerable therapeutic potential. However, as an essential protein with complex regulation and tissue specificity^{50,52}, Drp1 requires further studies to avoid adverse effects during therapy.

1.4 Miro1

Mitochondrial transport is mediated by the atypical Rho GTPase Miro¹⁶¹ which is conserved in most eukaryotes^{161,162}. Humans have two Miro proteins; Mitochondrial Rho GTPase 1 and 2 (Miro1 and Miro2 encoded by *RHOT1* and *RHOT2*, respectively) which share ~60% sequence homology and are ubiquitously expressed¹⁶¹.

Miro1, a protein of ~70 kDa^{161,163} with four splice variants¹⁶⁴, is localized to both mitochondria and peroxisomes¹⁶⁴. Besides its role in mitochondrial transport, Miro1 is involved in calcium signalling and mitochondrial quality control³⁴.

Miro1 structure

Miro1 is anchored to the MOM via a C-terminal transmembrane domain with the bulk of the protein oriented towards the cytoplasm^{47,161}. It contains two distinct GTPase domains¹⁶¹: the N-terminal and the C-terminal GTPase, with the latter lacking characteristic Ras residues and being more promiscuous hydrolysing ATP and UTP as well^{165,166}. The GTPase domains flank ELM1 and ELM2¹⁶⁷, each consisting of two canonical EF-hand domains accompanied by a “hidden” EF-hand domain^{161,166} (**Figure 4**). Via these EF-hands, Miro can sense both cytosolic and mitochondrial Ca²⁺ levels^{168,169}.

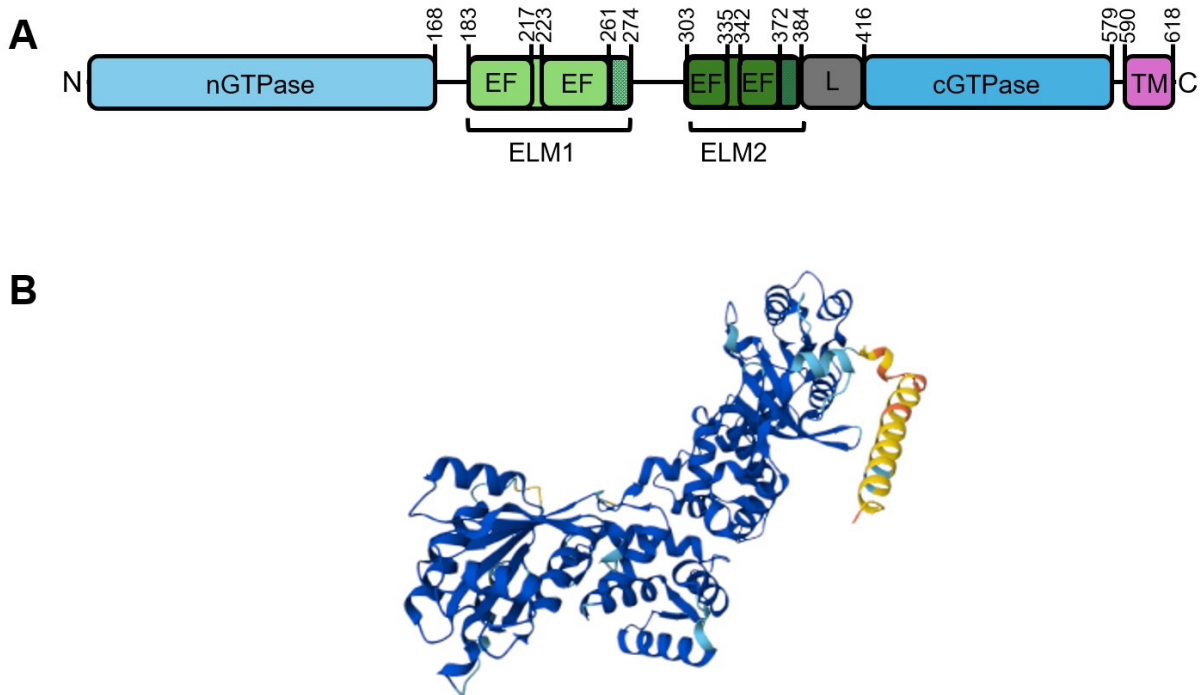


Figure 4: Miro1 structure

A Schematic illustration of Miro1 showing N-terminal and C-terminal GTPase (nGTPase and cGTPase, respectively), the two EF-hand pairs (ELM1 and 2), linker region (L) and transmembrane domain (TM). Domain borders are indicated according to Ravitch *et al.*¹⁶⁷. **B** AlphaFold prediction of Miro1 isoform1 coloured according to model confidence (dark blue: very high (pLDDT>90), light blue: high (pLDDT >70), yellow: low (pLDDT >50), orange: very low (pLDDT<50)). Structure is taken from the AlphaFold Protein Structure Database with a model creation date of 1 Aug 2025^{72,73}.

Miro1 is a key player in mitochondrial transport that is regulated by calcium signalling

Miro is a central player for mitochondrial transport along microtubules as it connects mitochondria and kinesin motor proteins in concert with its adaptors trafficking kinesin-binding protein 1 and 2 (TRAK1 and TRAK2)⁴⁷. Miro1 interacts with TRAK1 at two sites, at the ELMs and C-terminal GTPase and in the cleft between N-terminal GTPase and ELM1^{167,170}. Together, Miro1 and TRAK1 form a hetero tetramer¹⁶⁷.

Although the role of TRAK1 and 2 as adaptors is established, it has been debated if they are required for the interaction between Miro and the motor proteins^{171,172}. MacAskill *et al.* showed that Miro1 could bind to KIF5 motors as they co-immunoprecipitated independently of TRAK2¹⁷¹ (**Figure 5, left**). The same year, Wang

and Schwarz demonstrated that Miro requires the *Drosophila* adaptor milton for recruitment of the kinesin heavy chain (KHC) as in the absence of milton co-immunoprecipitation levels were low and fluorescence microscopy showed mitochondrial KHC localization only in the presence of milton¹⁷² (**Figure 5, right**). Considering that milton has only 35 % similarity to TRAK2, these contradicting results could be rooted in differences between *Drosophila* and mammals¹⁷¹.

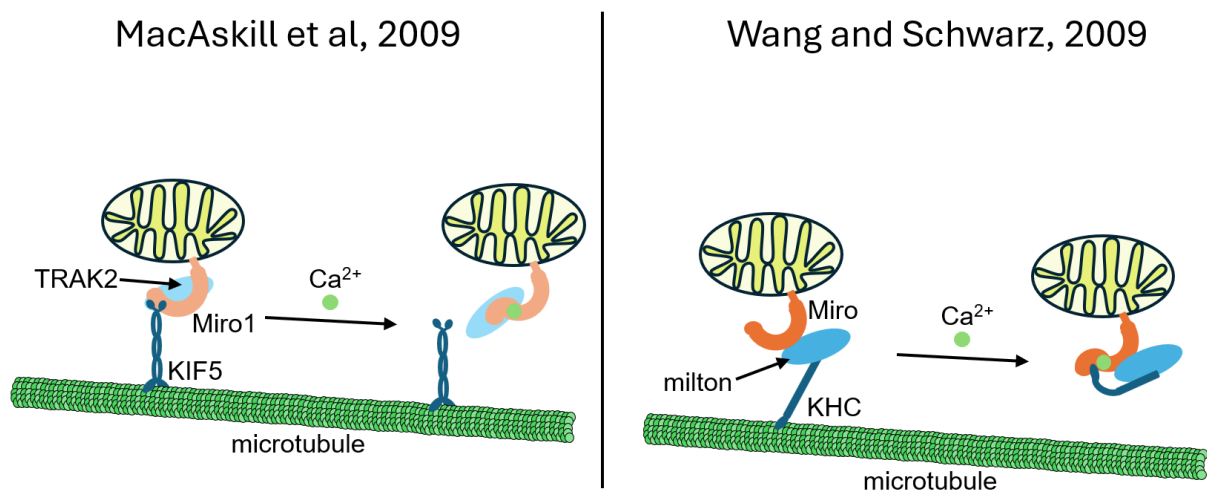


Figure 5: The two proposed models of Miro interaction with kinesins

Two models for Miro interaction with kinesins have been proposed. In the model by MacAskill et al., Miro1 directly interacts with KIF5 (left). Upon Ca^{2+} influx, a conformational change in Miro1 results in KIF5 detachment from Miro1. Wang and Schwarz proposed a model in which the kinesin heavy chain (KHC) interacts with the Miro-binding protein milton (right). Ca^{2+} influx results in binding of the KHC to Miro thus leading to detachment of the KHC from the microtubule.

Mitochondria are transported to different cellular regions, e.g. axons and dendrites in neurons. Van Spronsen et al. showed that mitochondrial transport to axons is mediated by TRAK1, which binds both kinesin-1 and dynein/dynactin, while the predominantly dynein/dynactin binding TRAK2 mediates dendritic transport¹⁷³ which allows independent regulation of the two opposing transport processes.

Miro function is regulated by Ca^{2+} signalling^{169,171,172}. Upon increase of cytosolic $[\text{Ca}^{2+}]$, Miro mediates the arrest of mitochondrial movement^{169,174} either by dissociation of KIF5 from Miro1¹⁷¹ or by Miro-mediated KHC dissociation from microtubules¹⁷²

(Figure 5). This allows the positioning of mitochondria in high energy demand regions where Ca^{2+} influx occurs^{169,171,172}.

Miro1 in mitochondrial quality control

To maintain a pool of healthy mitochondria, dysfunctional organelles must be recognized and degraded which is especially important in non-dividing cells like neurons as they cannot dilute out damaged mitochondria by cell division. A key pathway for mitochondrial degradation by mitophagy is the PINK1-Parkin axis. In healthy cells, PINK1 is localized to mitochondria where it is constantly degraded. When mitochondrial membrane potential drops, PINK1 is no longer degraded and accumulates on the MOM^{34,175}. As a kinase, PINK1 starts to phosphorylate its targets leading to activation of the E3 ligase Parkin which ubiquitinates MOM proteins resulting in recruitment of autophagy-mediating proteins and ultimately mitophagy^{107,176-179}.

Miro1 is a target of the PINK1/Parkin axis^{177,180} **(Figure 6)**. Phosphorylation of Miro1 at Ser156 by PINK1 activates Parkin which subsequently ubiquitinates Miro1^{176,177,181}. Miro1 and Parkin already interact at basal conditions but ubiquitination of Miro1 only occurs upon activation¹⁸². In addition, Parkin has a higher affinity for Miro1 compared to its other substrates¹⁸¹. The resulting early degradation of Miro1 was hypothesized to facilitate mitophagy by arresting damaged mitochondria and confining the potential release of damaging reagents like ROS to a smaller area^{177,181}.

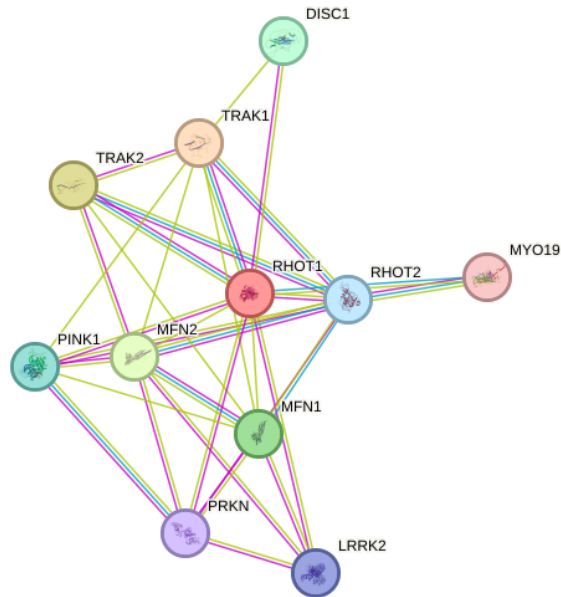


Figure 6: Miro1 interactors according to STRING database

Miro1 (RHOT1) first shell of physical interactors as visualised by STRING database (as of 20.2.2026)^{183,184}. Lines are colour-coded according to the source of information (blue: from curated databases, pink: experimentally determined, green: textmining).

Miro1 is localized to both mitochondria and peroxisomes

Miro1 is dually localized to mitochondria and peroxisomes¹⁶⁴. From the four Miro1 splice variants, variant2 and variant4 have been reported to be partially or mainly localized to peroxisomes¹⁶⁴, while for variant3 the peroxisomal localisation is debated^{164,185}. The function of Miro1 in peroxisomes, however, remains elusive. Miro1 interacts with Pex19^{164,185}, an import receptor for peroxisomal membrane proteins¹⁸⁶⁻¹⁸⁸. While one study found Miro1 being involved in peroxisomal movement¹⁶⁴, a different study could not replicate this and instead suggested a negative regulation of Drp1-mediated peroxisomal fission, a function that is shared with mitochondria¹⁸⁵.

Miro1 in altered in neurodegenerative diseases

While mutations in *PINK1* and *PRKN* (encoding for PINK1 and Parkin, respectively) have been associated with PD more than two decades ago^{189,190}, *RHOT1* mutations have only recently been linked to PD^{191,192}.

More interestingly, the failure to degrade Miro1 during mitophagy is emerging as a unifying element in PD. PD-patient-derived fibroblast cell lines and induced pluripotent stem cells (iPSCs) were unable to remove Miro1 after carbonyl cyanide m-chlorophenylhydrazone (CCCP)-induced mitochondrial depolarization while fibroblasts from healthy controls and from patients with other neurodegenerative diseases could clear Miro1. Thus, Miro1 retention could be used as a diagnostic marker for PD¹⁹³⁻¹⁹⁵. In addition, Miro1 modulation might be a feasible therapeutic approach. Therefore, Hsieh *et al.* screened a small molecule database for Miro1 binders and identified a compound which promoted Miro1 degradation in PD-patient-derived fibroblasts. This compound with non-disclosed identity was named “Miro1 Reducer” and could rescue the loss of dopaminergic neurons in a fly model¹⁹³. Therefore, Miro1 is an emerging target for both diagnostics¹⁹⁵ and therapeutic interventions^{193,194}.

In addition, Miro1 has been linked to amyotrophic lateral sclerosis (ALS). ALS is characterized by motor neuron degeneration and has multiple phenotypes like weakness of upper and lower limb muscles and frontotemporal dementia. While the cause is unknown in most cases, about 2% of patients carry mutations in the superoxide dismutase gene (*SOD1*) causing mitochondrial dysfunction^{196,197}. Another ALS-associated protein is trans-activating response region DNA-binding protein 43 (TDP-43) which regulates mRNA biogenesis and binds to mitochondrial tRNAs¹⁹⁸. In mice carrying ALS-related *SOD1*- or TDP-43-mutations, Miro1 levels were reduced in the spinal cord but not in brain tissue¹⁹⁹. In addition, an ALS-associated mutation in vesicle-associated membrane protein-associated protein B (VAPB), which functions in Ca²⁺ regulation between ER and mitochondria, disrupts anterograde mitochondrial transport by affecting the Miro1/kinesin-1/tubulin interaction²⁰⁰. Together with previous reports of impaired mitochondrial transport in ALS²⁰¹⁻²⁰³, this indicates a role of Miro1 in ALS-pathology.

1.5 Nanobodies

Common protein labelling methods are limited

The ability to specifically target the protein of interest (POI) is crucial for many cell biology and biochemical methods. For instance, fluorescence microscopy requires a fluorescent emitter. In live cell microscopy, this is often achieved by tagging the POI with a fluorescent protein like green fluorescent protein (GFP)²⁰⁴⁻²⁰⁷. However, the addition of such a large protein can influence the properties and behaviour of the POI²⁰⁸⁻²¹⁵. GFP-tagging of Drp1, for example, alters both its oligomerization and GTPase activity²¹⁶.

Another common approach for POI detection is the use of antibodies (Abs). While targets exposed on the outer surface of the plasma membrane are accessible for Abs²¹⁷⁻²¹⁹, staining of intracellular targets requires the fixation and permeabilization of cells as Abs cannot cross the plasma membrane. Furthermore, intracellular expression is not possible because Abs cannot be properly folded in the cytoplasm due to their heterotetrameric structure and disulfide bridges, which require an oxidizing environment²²⁰. To overcome these limitations, smaller formats have been developed that consist only of the paratope-forming variable domains of the heavy and light chains, such as single-chain variable fragments (scFvs)²²¹⁻²²³. However, scFvs are prone to aggregation due to the exposure of hydrophobic amino acid residues on their surface, which makes their intracellular application challenging^{220,224}.

Heavy-chain only antibodies

Based on a serendipitous discovery more than 30 years ago, a new class of antibody-derived binders was developed. In 1993, Hamers-Casterman *et al.* discovered functional heavy-chain only antibodies (HCAbs) in camels and other camelids like llamas and alpacas²²⁵. In HCAbs, the C_H1 domain, which connects the constant domain of the light chain with the heavy chain, is removed due to a splice site-located G to A mutation²²⁵⁻²²⁷ (**Figure 7A**). To compensate for the lack of light chains, camelid HCAbs have several alterations. For instance, four amino acid residues in framework region 2 that usually make contact with the light chain have been replaced with smaller and/or hydrophilic amino acids (i.e. Val⁴² to Phe or Tyr, Gly⁴⁹ to Glu, Leu⁵⁰ to Arg or Cys, Trp⁵² to Gly or Phe) thus improving solubility²²⁸⁻²³⁴. In addition, the comparatively long

complementarity-determining region 3 (CDR3) can fold back to the framework region further masking the light chain binding site²³⁵.

The variable domain of HCAbs (VHH) alone is sufficient for antigen binding making it one of the smallest immunoglobulin-derived binding fragments (~15 kDa) (**Figure 7A**)^{233,236,237}. Due to their small size, VHHs were dubbed “nanobodies” by the company Ablynx in 2001, a term that is now widely used by the scientific community to refer to VHHs in general^{220,232,238-243}.

As with any variable antibody fragment, nanobodies (Nbs) consist of three complementarity-determining regions (CDR1-3), which are surrounded by four framework regions (FR1-4)^{232,244} (**Figure 7B**). The binding mode of Nbs differs from that of classical Abs, which mainly recognize flat surfaces, as Nbs tend to bind structurally defined epitopes such as concave regions^{235,245}. The majority of Nbs bind either primarily with the CDR3 or equally utilize CDR and FR residues^{235,245-247}. Besides a conserved canonical disulfide bridge (Cys²² to Cys⁹²)²³³, Nbs that display an elongated CDR3 often comprise an additional disulfide bond between CDR3 and CDR1, FR2, or CDR2^{229,234,240,248-250}.

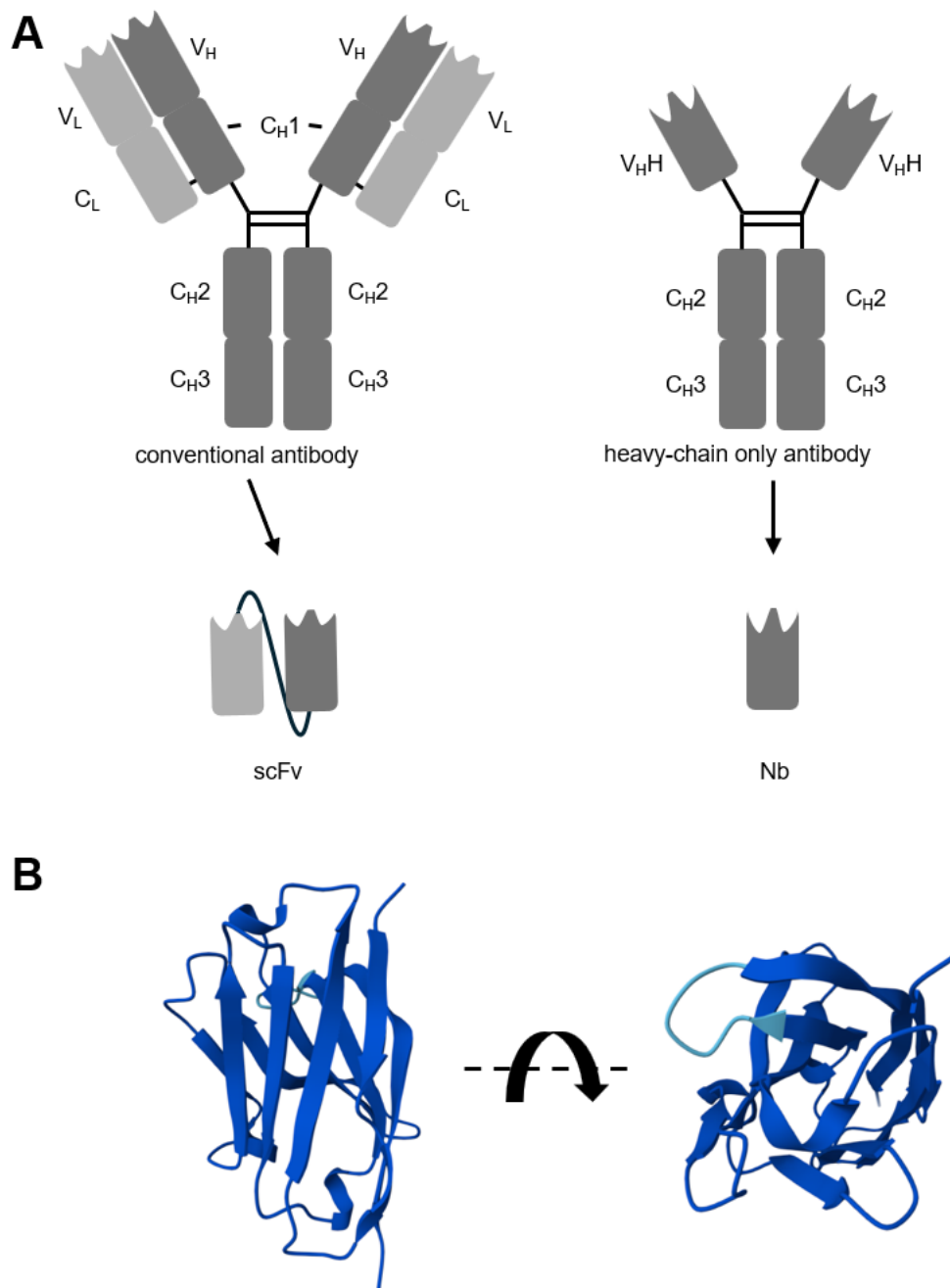


Figure 7: Antibodies and antibody-derived fragments

A Schematic depiction of conventional antibodies and heavy-chain only antibody and the respective derived fragments single-chain variable fragment (scFv) and nanobody (Nb). **B** AlphaFold3 modelling²⁵¹ of a Nb (D7) coloured according to model confidence (dark blue: very high (pLDDT>90), light blue: high (pLDDT >70)).

Nanobody generation and selection

Nbs can be selected from three different types of libraries: immune, naïve and synthetic libraries. For an immune library, a camelid is immunized via a series of antigen injections, followed by the isolation of B-lymphocytes from peripheral blood or from a lymph node biopsy^{220,252,253}. After mRNA extraction and cDNA synthesis, the Nb repertoire can be amplified by PCR to subsequently generate a recombinant Nb gene library. Afterwards, antigen-specific Nbs can be selected using different techniques, with phage display²⁵⁴, yeast display²⁵⁵ and ribosome display²⁵⁶ being the most common. Once target-specific Nbs have been selected, they can be produced at comparatively low costs in simple heterologous expression systems such as *E.coli*^{246,257}.

Nbs have a high affinity with equilibrium dissociation constants routinely reaching the low nanomolar or even sub-nanomolar range²³². In addition, Nbs are generally highly stable, as they usually denature above 60 °C and can be stored for months at 4 °C^{232,258}. As they also show good solubility and low immunogenicity^{232,257,259,260}, Nbs are well suited for a range of applications.

Applications of Nanobodies

Due to their beneficial properties, Nbs have been used for a wide range of applications. In structural biochemistry, for instance, Nbs are applied as chaperones or stabilizers during crystallography²⁶¹⁻²⁶⁴ and as fiducial markers in cryo-EM²⁶⁵⁻²⁶⁸.

Nbs are also employed for protein-protein interaction studies as they can easily be functionalized into affinity matrices for protein precipitations. The first and best-known of these capturing tools is the GFP-trap consisting of a GFP-Nb covalently coupled to Sepharose beads. This enables the pulldown of GFP-fusion proteins and their interacting partners which can then be further analysed, e.g. by mass spectrometry²⁶⁹. Similar Nb-based traps have been developed against other protein tags like RFP^{270,271} or peptide tags such as the BC2(SPOT)-tag²⁷² or the Pep-Tag²⁷³.

Super-resolution microscopy (SRM) methods like stimulated emission depletion microscopy (STED) and stochastic optical reconstruction microscopy (STORM) allowing resolutions below the diffraction limit of light have revolutionized microscopy. With the technically achievable resolution reaching now ~10 nm²⁷⁴, the linkage error (distance between epitope and fluorophore) of ~30 nm due to the size of a conventional

Ab becomes a limiting factor^{275,276}. Here, the smaller Nbs are an attractive alternative and have been successfully applied in various applications such as STED^{241,277-280}, STORM²⁸¹⁻²⁸⁵ and DNA-based point accumulation for imaging in nanoscale topography (DNA-PAINT)^{279,280,286}.

For tag-free antigen imaging in live cells, Nbs which are functional in the reducing environment of the cytoplasm have been genetically coupled to fluorescent proteins (FPs) such as GFP, generating so-called chromobodies (Cbs)²⁸⁷. A Cb-encoding plasmid can be then either transiently transfected²⁸⁷ or used to generate a stable cell line^{253,288}. With a molecular weight of ~40 kDa, Cbs can access different cellular compartments like the cytoplasm^{253,289} or nucleus²⁸⁸ and can be used in whole organisms like zebrafish embryos²⁸⁹ or mice²⁹⁰. Due to their relatively transient binding mode, Cbs have been reported to affect the function and dynamic distribution of their antigens less than other techniques²⁹¹⁻²⁹³. However, as the cellular expression of Cbs is difficult to adjust on the DNA level, signals from unbound Cbs may mask those from bound Cbs. Therefore, several strategies have been developed to ameliorate the signal-to-noise ratio creating conditionally stable²⁹⁴ or turnover-accelerated²⁹⁵ Cbs. The latter exploits that Cbs are stabilized in the presence of their antigen, a phenomenon termed antigen-mediated Cb stabilization (AMCBS)²⁹⁵. To utilize this, the Cb turnover was increased by applying the N-end rule, which states that the N-terminal amino acid residue determines the turnover and thus the cellular half life of a protein^{296,297}. When testing the turnover of Cbs with different N-terminal amino acids, Cbs displaying an arginine (Arg) showed a faster turnover in the absence of their antigen²⁹⁵. The presentation of an N-terminal Arg was hereby achieved using a Cb expression construct comprising an N-terminal Ubiquitin (Ub) followed by an Arg as the first amino acid residue of the Cb²⁹⁵. Upon co-translational cleavage of Ub, Arg becomes N-terminally exposed and the Cb is subjected to preferred degradation by the proteasomal system²⁹⁵. Notably, antigen binding hinders degradation, resulting in the stabilization of the antigen-bound Cb, which confers an improved signal-to-noise ratio²⁹⁵. Turnover-accelerated Cbs can be used not only to visualize endogenous antigens in live cells, but also to determine and quantify changes in the levels of intracellular antigens^{273,294,295}. However, it has to be considered that not all Nbs are intracellularly functional. Thus, cysteine-less Nb libraries²⁹⁸ or targeted mutagenesis²⁴⁸ have been used to generate suitable binding molecules.

Nbs can also be used for targeted protein degradation. Methods like CRISPR/Cas9 and RNA interference are widely used for knockdowns or knockouts. However, these have limitations when working with essential or long-half live proteins²⁹⁹. Thus, Nbs have been successfully applied to redirect the cellular protein degradation machinery to the POI. The best-known approach is the deGradFP system that utilizes the F-box domain of *Drosophila* Slimb fused to a GFP-binding Nb to recruit SCF E3 ubiquitin ligases for GFP-fusion-protein degradation³⁰⁰. Since its development, deGradFP has been widely adopted³⁰¹⁻³⁰⁶. An alternative approach utilizes TRIM21, an E3 ubiquitin ligase recognizing the Fc domain of antibodies and redirecting them and their cargo, e.g. bacterial or viral proteins, for proteasomal degradation³⁰⁷⁻³⁰⁹. Originally applied in combination with electroporation or microinjection of antibodies, the method was further developed by directly fusing a target-binding Nb with the RING domain of TRIM21^{309,310}. As clustering of the RING domain is required for degradation³¹¹, either the POI needs to oligomerize on its own or the B-box and coiled-coil domains of TRIM21 are added to the construct (abbreviated as RBCC)³¹². Besides deGradFP and TRIM21, other Nb-based degron systems have been developed based on the adaptor proteins speckle-type poxvirus and zinc finger domain protein (SPOP)³¹³ and the plant-derived TIR³¹⁴.

In recent years, Nbs have also reached the clinics for both therapeutic and diagnostic applications. In 2018, Caplacizumab became the first EMA-approved Nb with FDA-approval following in 2019³¹⁵. This humanized bivalent Nb targeting von Willebrand factor is indicated for the treatment of immune-mediated thrombotic thrombocytopenic purpura^{315,316}. Since then, other therapeutics have reached market³¹⁶ or are currently in clinical trials³¹⁷⁻³¹⁹ including nanobody-based chimeric antigen receptor (CAR)-T cells³²⁰. In addition, Nbs are tested for diagnostic purposes^{239,321-323}. For instance, human epidermal growth factor receptor 2 (HER2) status in breast cancer is relevant for both prognosis and choice of treatment³²². However, biopsies can be misleading due to heterogenous HER2 expression³²². Here, radiolabeled anti-HER2 Nbs, due to their fast tissue penetration, enable same-day non-invasive whole-body imaging of patients as demonstrated in both phase I and II clinical trials^{239,322,324}. Additionally, to monitor the immune response upon cancer treatment, Nbs against immune cell markers like CD8³²⁵, CD4^{326,327} and OX40³²⁸ have been developed.

In conclusion, Nbs are not only a versatile research tool but also have the potential to become widely used for both therapeutic and diagnostic clinical applications (**Figure 8**).

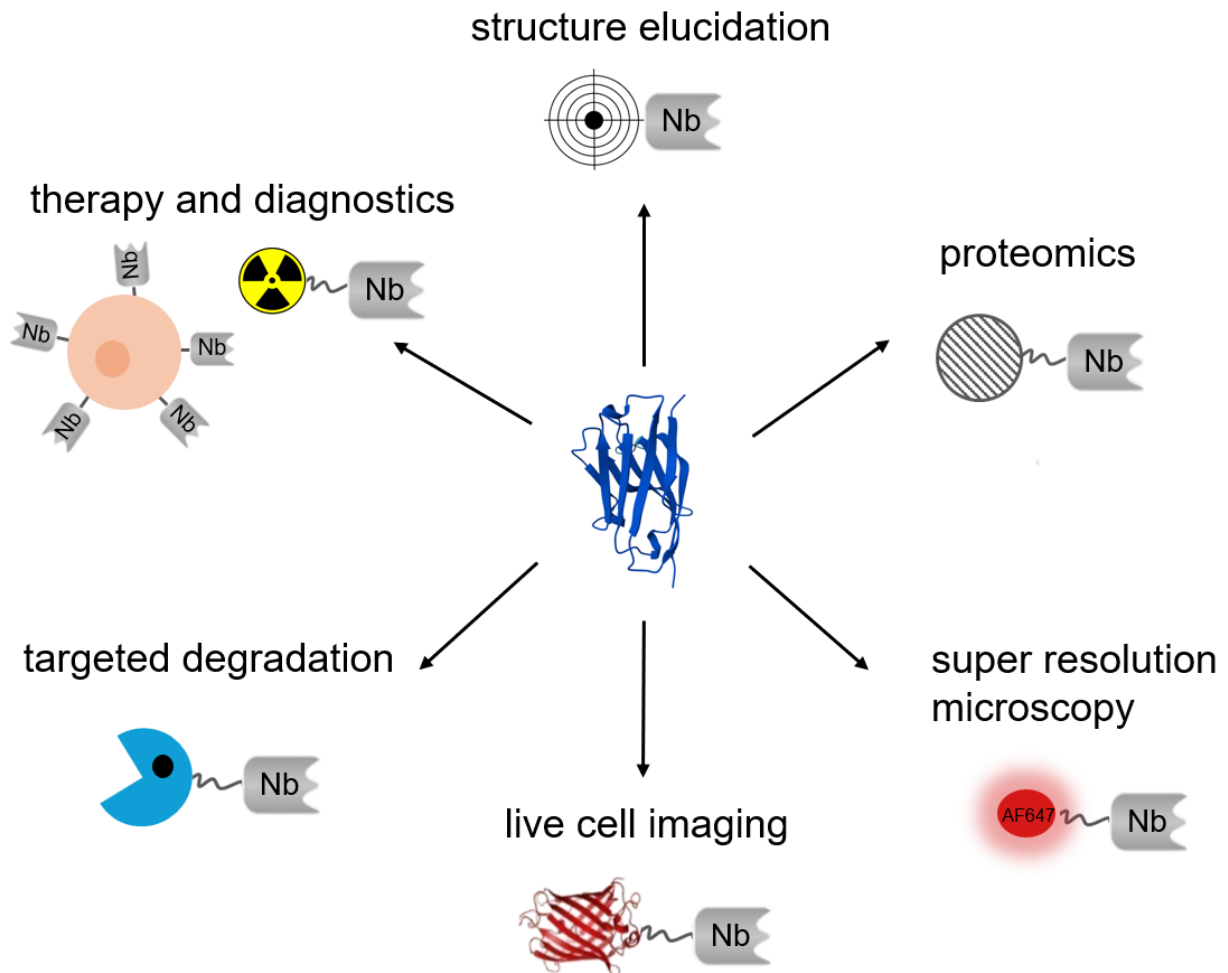


Figure 8: Nanobodies can be functionalized for different applications

Nbs are applied as reference points in cryo-EM for structural elucidation. Immobilized on agarose beads, Nbs function as affinity-capture tools allowing subsequent proteomic analyses. For imaging applications, Nbs can be coupled to fluorescent dyes for super resolution microscopy or expressed as fluorescent protein-fusions for live cell imaging. Coupled with a degradation domain, Nbs enable the targeted degradation of the protein of interest. Nbs have been applied as therapeutics, e.g. as CAR-T cells, or as radiolabelled tracers for diagnostic purposes. (Nb structure: see Figure 7B).

1.6 Cellular transduction of proteins

Biopharmaceuticals, which encompass recombinant proteins, nucleic acid- and genetically engineered cell-based products, are a rising therapeutical class with global annual sales estimated at over 300 billion US-dollars and over 1000 phase III clinical trials in 2021³²⁹. Notably, most biologicals are currently developed against targets located on the cellular surface as the cellular plasma membrane is impermeable for proteins thus making the intracellular space inaccessible for protein-based therapeutics³³⁰. Therefore, intracellular protein delivery has a huge pharmaceutical potential considering that ~80 % of the human genome is deemed undruggable by small molecules^{330,331}. In the last decades, a plethora of protein delivery methods have been developed which can be classified into three categories: physical methods, direct protein engineering and nanocarrier-based delivery³³⁰.

Physical methods like electroporation, cell squeezing or microinjection show high efficiencies on single cell level but lack therapeutical feasibility due to being low throughput (e.g. microinjection) or being limited to *ex vivo* applications³³⁰.

Alternatively, cargo proteins can be combined with peptides derived from viral or bacterial proteins that are able to cross the plasma membrane³³⁰. As first cell penetrating peptide (CPP), a peptide from the Tat protein of HIV was used enabling the delivery of different cargos like horseradish peroxidase or β -galactosidase into different cell types³³². Since then, a wide range of CPP classes have been developed including cyclic and multimeric CPPs³³⁰. However, the efficiency varies widely and can also depend on cargo size and cell line^{330,333}. CPPs are often prone to endosomal entrapment, can inhibit the cargo function or might trigger immune responses^{330,333}. These issues are underscored by the lack of FDA-approved CPP-based applications³³⁰.

Alternatively, the cargo protein can also be embedded in nanocarriers like liposomes, lipid nanoparticles, gold nanoparticles or exosomes. Due to their size, nanocarriers have often low tissue penetration and methods for efficient packaging like repeated freeze-thaw cycles can damage the cargo protein³³⁰.

Thus, despite substantial efforts, there is currently no broadly applicable protein delivery method available thus sparking the development of new methods.

In 2020, Tai *et al.* published a new approach for intracellular protein delivery by fusing cholesterol to coomassie blue³³⁴. Coomassie binds noncovalently to proteins while

cholesterol, as compound of the plasma membrane, can integrate into membranes thus allowing the then-shielded cargo to enter the cell (**Figure 9**). The authors demonstrated efficient cytoplasmic delivery of several proteins without endosomal entrapment including enzymatically functional horseradish peroxidase³³⁴. As this method worked was described to work especially well for small, compact proteins, we hypothesized that it might be a promising approach to transduce Nbs.

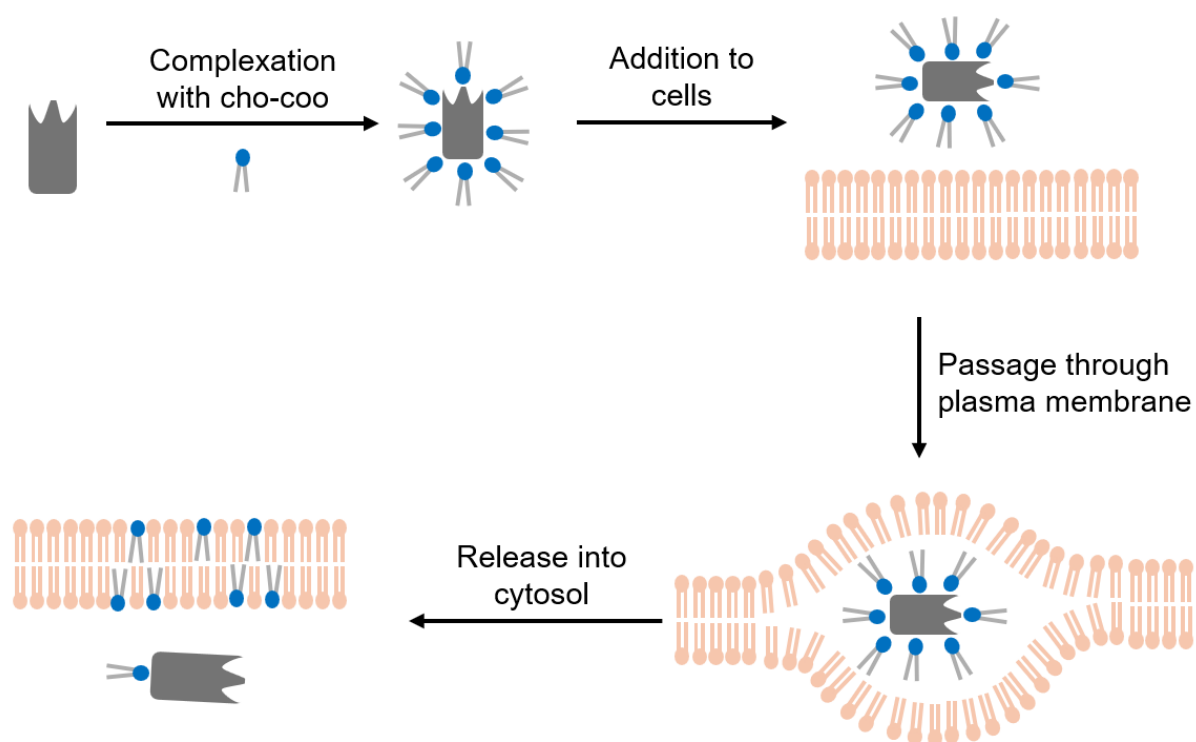


Figure 9: Schematic depiction of the proposed mode of action of cholesterol-coomassie

The cargo protein is complexed with cholesterol-coomassie before addition to the to-be-transduced cells. The outward-facing cholesterol can then integrate into the plasma membrane, thus allowing passage and subsequent release of the cargo protein into the cytoplasm. Figure based on Tai et al.³³⁴.

1.7 Aims and objectives of this study

While the MOM is central for mitophagy, mitochondrial movement, organelle shape and many other mitochondrial functions, it is currently understudied. Therefore, the Deutsche Forschungsgemeinschaft (DFG)-funded research training group “MOMbrane” aims to further investigate different aspects of the MOM including its function and regulation. In this project, our group focuses on the selection, functionalization and application of Nbs against the MOM proteins Miro1 and Drp1 thus providing new research tools to study mitochondrial dynamics. In the first funding period, Nbs against Miro1 and Drp1 were selected and initially characterized by F. Fagbadebo with emphasis on Miro1³³⁵. Consequently, in this work, I focused on the Drp1 Nbs.

Therefore, the main objective was to optimize the binding properties of the previously generated Drp1-Nbs and functionalise them for application in proof-of-principle studies. Hereby, I focused on intracellular applications. To improve binding of the Drp1-Nbs, the avidity was increased by generating bivalent formats to be applied in super resolution microscopy. In addition, the Drp1-Nbs were functionalized and tested for application as affinity-capture tools, for live cell microscopy and targeted protein degradation.

In addition, to finalise the works on Miro1-Nbs, we aimed improve the Miro1-Nb-based affinity capture tools (Miro1-traps). For this, in collaboration with F. Fagbadebo, I generated a bivalent format of the lead candidate M114 which was functionalised into a Miro1-trap to be subsequently applied in a proof-of-principle Miro1 interactome study.

Finally, I aimed to establish a general protocol for intracellular delivery of Nbs into live cells. For this, I tested the previously published cholesterol-coomassie (cho-coo) protein transduction method³³⁴ for intracellular delivery of Nbs.

2 Materials and Methods

2.1 Materials

Chemicals and Media	Manufacturer
2-mercaptoethanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
2-Propanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acetic acid	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acetone	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acrylamide/Bisacrylamide	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agarose, molecular biology grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ammonium persulfate (APS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ampicillin	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DAPI (4,6-diamidino-2-phenylindole)	Roche diagnostics GmbH, Mannheim, Germany
cholesterol-coomassie	kindly provided by Xiaohu Gao (University of Washington, USA) ³³⁴
DBCO-AF488	Jena Bioscience, Jena, Germany
DBCO-AF647	Jena Bioscience, Jena, Germany
DBCO-agarose beads	Jena Bioscience, Jena, Germany
Disodium hydrogen phosphate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DNA loading dye (6x)	New England Biolabs, Frankfurt, Germany
DMEM +Glutamax, high glucose, pyruvate	Thermo Fisher Scientific GmbH, Schwerte, Germany
DMEM/F12 media	Thermo Fisher Scientific GmbH, Schwerte, Germany
DMSO	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DNaseI	Applichem GmbH, Darmstadt, Germany
dNTP solution mix	New England Biolabs, Frankfurt, Germany
Doxycycline	Sigma-Aldrich, Taufkirchen, Germany
Dulbecco's phosphate buffered saline (PBS)	Thermo Fisher Scientific GmbH, Schwerte, Germany
Ethanol, HPLC grade	Sigma-Aldrich, Taufkirchen, Germany
Ethanol, denatured	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethylene diamine tetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Fetal bovine serum (FBS)	Thermo Fisher Scientific GmbH, Schwerte, Germany
Fluorobright Media	Thermo Fisher Scientific GmbH, Schwerte, Germany
Formaldehyde (37%)	Sigma-Aldrich, Taufkirchen, Germany
Glycine	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Glycerol	Sigma-Aldrich, Taufkirchen, Germany
H-Gly-Gly-Gly-propyl-azide	AG Maurer, University Tübingen
H-Gly-Gly-Gly-Arg-Arg-Lys-(N ₃)-NH ₂	AG Schwarzer, University Tübingen
Hoechst33342	Thermo Fisher Scientific GmbH, Schwerte, Germany
Hoechst33258	Thermo Fisher Scientific GmbH, Schwerte, Germany
Hydrochloric acid 37%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Isopropyl-beta-D-thiogalactopyranoside (IPTG)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Imidazole	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Instant Blue Coomassie	Abcam, Cambridge, UK
Kanamycin	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
LB media (Lennox)	Sigma-Aldrich, Taufkirchen, Germany
Lipofectamine 2000	Thermo Fisher Scientific GmbH, Schwerte, Germany
Lysozyme	AppliChem GmbH, Darmstadt, Germany

Magnesium chloride hexahydrate	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
Methanol	Sigma-Aldrich, Taufkirchen, Germany
Midori Green	NIPPON Genetics EUROPE GmbH, Düren, Germany
Milk powder	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
MitoTracker green	Cell Signaling Technology, Frankfurt, Germany
Mycoplasma PCR Detection Kit	Abm, Richmond, Canada
NHS-activated Sepharose 4 Fast Flow	Cytiva Europe GmbH, Freiburg im Breisgau, Germany
NucleoSpin Gel and PCR Clean-Up kit	Macherey-Nagel, Düren, Germany
NucleoSpin Plasmid kit	Macherey-Nagel, Düren, Germany
Nuclease-free water	New England Biolabs, Frankfurt, Germany
OptiMEM	Thermo Fisher Scientific GmbH, Schwerte, Germany
PageRuler Plus Prestained Protein ladder	Thermo Fisher Scientific GmbH, Schwerte, Germany
Paraformaldehyde (PFA)	Merck KGAA, Darmstadt, Germany
Penicillin/Streptomycin (100x)	Thermo Fisher Scientific GmbH, Schwerte, Germany
Phenylmethanesulfonylfluoride (PMSF)	SERVA Electrophoresis GmbH, Heidelberg, Germany
phosphate buffered saline (PBS), tablets	Thermo Fisher Scientific GmbH, Schwerte, Germany
Polyethyleneimine (PEI)	Sigma-Aldrich, Taufkirchen, Germany
Ponceau S	Carl Roth GmbH &Co. KG, Karlsruhe, Germany
Primers and gene synthesis	Integrated DNA technologies, Leuven, Belgium
Protease Inhibitor Mix B and Mix M	SERVA Electrophoresis GmbH, Heidelberg, Germany
Protein A/G beads	Cytiva Europe GmbH, Freiburg im Breisgau, Germany
Q5 Polymerase and buffer, 2x MasterMix Q5	New England Biolabs, Frankfurt, Germany
restriction enzymes and buffer	New England Biolabs, Frankfurt, Germany
SOC medium	New England Biolabs, Frankfurt, Germany
Sodium chloride (NaCl)	Carl Roth GmbH &Co. KG, Karlsruhe, Germany
Sodium dodecyl sulphate (SDS)	Carl Roth GmbH &Co. KG, Karlsruhe, Germany
Sodium hydroxide	Carl Roth GmbH &Co. KG, Karlsruhe, Germany
Sodium azide	Carl Roth GmbH &Co. KG, Karlsruhe, Germany
Sortase	In house production
Sulfo-NHS-LC-LC-Biotin	Thermo Fisher Scientific GmbH, Schwerte, Germany
T4 Ligase and T4 Ligase buffer	New England Biolabs, Frankfurt, Germany
TB media	Carl Roth GmbH &Co. KG, Karlsruhe, Germany
TEMED (N'-tetramethylethylenediamine)	Carl Roth GmbH &Co. KG, Karlsruhe, Germany
Tris (trisaminomethane)	Carl Roth GmbH &Co. KG, Karlsruhe, Germany
Trypsin-EDTA (0.25%), with phenol red	Thermo Fisher Scientific GmbH, Schwerte, Germany
Tween20	Carl Roth GmbH &Co. KG, Karlsruhe, Germany

Bacteria and mammalian cell lines	Distributer/acquired from
10-beta <i>E. coli</i>	New England Biolabs, Frankfurt, Germany
XL1 blue competent <i>E.coli</i>	VWR, Darmstadt, Germany
A549 cells	ATCC
ExpiCHO cells	Thermo Fisher Scientific GmbH, Schwerte, Germany
HeLa Kyoto cells (Cellosaurus no. CVCL_1922)	acquired from S. Narumiya (Kyoto University, Japan)

HeLa BC2T ^{ACTB} cells	Virant <i>et al.</i> (2018) ²⁸⁵
HeLa Drp1 KO cells	generated in and provided by the lab of Thomas Langer (Max Planck Institute, Cologne, Germany)
HEK293 cells (CRL3216)	ATCC
U2OS (HTB-96)	ATCC

Antibodies	Dilution	Application	Manufacturer
Anti-Drp1 antibody (D6C7, rabbit, mAb)	1: 1000 in BSA	WB	Cell Signaling Technology, Frankfurt, Germany
	1:100-1:300 in BSA	IF	
Anti-GAPDH antibody (PA1-987, rabbit, pAb)	1:1000 in milk	WB	Thermo Fisher Scientific GmbH, Schwerte, Germany
Anti-penta-His antibody (mouse, mAb)	1:1000 in milk	WB	Qiagen, Hilden, Germany
Anti-TagRFP (clone AB233; rabbit, pAb)	1:1000 in milk	WB	Evrogen, Moscow, Russia
Anti-rabbit antibody, AF488 labelled (goat)	1:1000 in milk	WB	Thermo Fisher Scientific GmbH, Schwerte, Germany
	1:1000 in BSA	IF	
Anti-mouse-AF647 antibody (goat)	1:1000 in milk	WB	Thermo Fisher Scientific GmbH, Schwerte, Germany
Anti-VHH-Cy5 antibody (goat)	1:500 in BSA	IF	Jackson Immuno-Research, Ely, UK

Plasmids	Reference
pHEN6C	Rothbauer <i>et al.</i> (2008) ²⁶⁹
GBP in pHEN6C	Rothbauer <i>et al.</i> (2006) ²⁸⁷
D7 in pHEN6C	F. Fagbadebo, ^{335,336}
D63 in pHEN6C	F. Fagbadebo, ^{335,336}
PepNb in pHEN6C	Traenkle <i>et al.</i> (2020) ²⁷³
bivBC2 in pHEN6C	Virant <i>et al.</i> (2018) ²⁸⁵
bivVB6 in pHEN6C (KKK and LPETG versions)	Maier <i>et al.</i> (2015) ²⁵³ and Virant <i>et al.</i> (2018) ²⁸⁵
pCDNA3.4	Addgene
bivD7 in pCDNA3.4	This study
bivD63 in pCDNA3.4	This study
bivM114 in pCDNA3.4	This study, ³³⁵
pTagRFP	Panza <i>et al.</i> (2015) ²⁸⁹
D7 in pTagRFP	F. Fagbadebo, ^{335,336}
D63 in pTagRFP	F. Fagbadebo, ^{335,336}
Pep-Cb in pTagRFP	Traenkle <i>et al.</i> (2020) ²⁷³
bivD7 in pTagRFP	This study, ³³⁶
bivM114Cb in pTagRFP	Fagbadebo <i>et al.</i> (2022) ³³⁷
pEGFP-Ubi-R-3xGS-tagRFP	Keller <i>et al.</i> (2018) ²⁹⁵
D7 in pEGFP-Ubi-R-3xGS-tagRFP	F. Fagbadebo ³³⁵
D63 in pEGFP-Ubi-R-3xGS-tagRFP	F. Fagbadebo ³³⁵
pB-rtTA, Addgene #126034	Kirk <i>et al.</i> (2018) ³³⁸
pSBet-GP, Addgene #60495	Kowarz <i>et al.</i> (2015) ³³⁹
Dox-inducible vector	This study
pCDNA3_NSImb-vhhGFP4	Caussin <i>et al.</i> (2011) ³⁰⁰
GBP-Fbox-TagRFP	This study
PepNb-Fbox-TagRFP	This study
D7-Fbox-TagRFP	This study

D63-Fbox-TagRFP	This study
HLTV-hTRIM21, Addgene #104973	Clift <i>et al.</i> (2017) ³¹⁰
GBP-RBCC-TagRFP	This study
PepNb-RBCC-TagRFP	This study
D7-RBCC-TagRFP	This study
D63-RBCC-TagRFP	This study
AcGFP-Drp1	Jenner <i>et al.</i> (2022) ¹⁰⁸
pTYB2	Jenner <i>et al.</i> (2022) ¹⁰⁸
pEGFP-N1	Clontech
Drp1 in pEGFP-N1	F. Fagbadebo, ^{335,336}
eSrtA in pET29 (Addgene #75144)	A gift from David Liu, ³⁴⁰
pEGFP-C1	Clontech
BC2T-GFP-NLS in pEGFPN1	Virant <i>et al.</i> (2018) ²⁸⁵

Primers and gene synthesis	Sequence
#1133	GTTGTAAAACGACGGCCAGTG
#1244	caccaccgccagatccaccgccacctgatcctccgctccGCTGCTAACGGTAACCTG
#1263	tttaattaagcggccggaattGTTGTAAAACGACGGCCAGTG
#1289	GAAAcgtctcaactctCAGGTGCAGCTGGTGGAGTC
#1373	gccggcgtgcactctGAGGTaCAGCTGCAGGAGTCGGG
#1374	ggtggatctggcgggtggtggaagtgggtggcggaggtagtGAGGTaCAGCTGCAGGAGTCGGG
#1375	gtctgtgaccgccggcgtgcactctGAG
#1429	GAAAcgtctcaactctCAGGTGCAGCTGGTGGAGAG
#1430	caccaccgccagatccaccgccacctgatcctccgctccTGAGGAGACGGTGACCCG
#1431	ggtggatctggcgggtggtggaagtgggtggcggaggtagtCAGGTGCAGCTGGTGGAGAG
#1434	GAAAcgtctcaactctCAAGTGCAGCTGGTGGAGTC
#1435	ggtggatctggcgggtggtggaagtgggtggcggaggtagtCAAGTGCAGCTGGTGGAGTC
#1438	GAAAcgtctcaactctCAAGTGC
#1504	caccaccgccagatccaccgccacctgatcctccgctccGCTGCTAACGGTGACCCG
#2065	cTTAATTAAGGTAAGGGGTCCGCTATCTAGAC
#2066	cgAGATCTGCTAGCCTCAGAGGCCTTTTCGAGGGT
#2081	ctaGCTAGCACCATGGTGAGCAAGGGC
#2082	gatcatCCTGAGGTTACTTGTACAGCTCGTCCATGC
#2083	gGGCCCAGCCGGCCaccATGGCTCAAGTCCAAGTGGTGGAGTCTG
#2084	agaacctcctccaccGCTGGAGACGGTGACCTGG
#2085	CAGCggtggaggaggttctATGATGAAAATGGAGACTGACAAAA
#2086	gctacctccgccaccactccaccgcctccGAGGTGGCGGCCAGTCC
#2087	ggaggcgggtggaagtgggtggcggaggtagcATGGTGTCTAAGGGCGAAGAGC
#2088	ctaGTCGACTCAATTAAGTTTGTGCCCCAGTTT
#2089	cataCTCGAGaccATGGCTCAAGTCCAAGTGGTGGAGTCTG
#2090	CAGCggtggaggaggttctATGGCTTCAGCAGCACGC
#2091	gctacctccgccaccactccaccgcctccCTCACTCCTTTCCAGGACAATTATC
MCS synthesis	cTTAATTAAGCTAGCGGCCAGCCGGCGGTTACCCTCGAGCCTCAG GGTCGACAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATA GCATCACAATTTCAAAATAAAGCATTTTTTTCACTGCATTCTAGTTGT GGTTTGTCCAAACTCATCAATGTATCTTAAGATCTcg

Consumables	Manufacturer
Amersham Protein 0.45 µl Nitrocellulose	Cytiva Europe GmbH, Freiburg im Breisgau, Germany
Amicon Ultra centrifugal filters (4ml, 15ml), 3MWCO and 15MWCO	Merck Millipore, Darmstadt, Germany
Baffled flask, 5L	Gerätebau Ochs, Bovenden, Germany
Cell culture flasks (T75, T175)	Corning GmbH, Wiesbaden, Germany
Cell culture plates (P100)	Corning GmbH, Wiesbaden, Germany
Centrifugation tubes (15ml and 50ml)	Greiner-Bio-One, Frickenhausen, Germany
Cryo tubes	Greiner-Bio-One, Frickenhausen, Germany
Filtropure S 0.45 µm syringe filter	Sarstedt AG&Co., Nümbrecht, Germany
GeBAflex Midi Dialysis tubes	Scienova GmbH, Jena, Germany
Hi Load 26/600 Superdex 75 pg column	Cytiva Europe GmbH, Freiburg im Breisgau, Germany
HiPrep 26/10 Desalting column	Cytiva Europe GmbH, Freiburg im Breisgau, Germany
HisTrap FF column, 1 ml	Cytiva Europe GmbH, Freiburg im Breisgau, Germany
Multiwell plates: 6-, 12-, 24-, and 96-wells (sterile)	Corning GmbH, Wiesbaden, Germany
Octet Streptavidin (SA) biosensor	Sartorius AG, Göttingen, Germany
µclear 96-well plates	Greiner-Bio-One, Frickenhausen, Germany
P100 dish	Corning GmbH, Wiesbaden, Germany
Parafilm	Brand GmbH&Co KG, Wertheim, Germany
PCR Tubes	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
Petri dish	Sarstedt AG&Co. Nümbrecht, Germany
pH Indicator strips, pH 0-14	Merck KGaA, Darmstadt, Germany
Pipette tips (20µl, 200µl, 1ml)	Sarstedt AG&Co. Nümbrecht, Germany
Reaction tubes (1.5ml, 2ml)	Eppendorf AG, Hamburg, Germany
Reaction tubes, low binding 1.5ml)	Eppendorf AG, Hamburg, Germany
Serological pipettes (2ml, 5ml, 10ml, 25ml, 50ml)	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
Slide-A-Lyzer	Thermo Fisher Scientific GmbH, Schwerte, Germany
Syringes (1 ml, 5 ml, 10 ml, 20 ml, 50 ml)	B. Braun Melsungen AG, Melsungen, Germany
Zeba-spin desalting columns	Thermo Fisher Scientific GmbH, Schwerte, Germany

Devices	Manufacturer
Äkta pure system	Cytiva Europe GmbH, Freiburg im Breisgau, Germany
Biometra Tone- PCR Thermocycler	Analytic Jena, Germany
BioPhotometer 6131 spectrometer	Eppendorf AG, Hamburg, Germany
Centrifuge 5424R	Eppendorf AG, Hamburg, Germany
Centrifuge 5702	Eppendorf AG, Hamburg, Germany
Centrifuge 5810R	Eppendorf AG, Hamburg, Germany
Centrifuge Heraeus Pico 21	Heaeus Instruments, Hanau, Germany
Centrifuge Multifuge X1R Pro	Thermo Fisher Scientific GmbH, Schwerte, Germany
Centrifuge Megafuge 1.0R	Heaeus Instruments, Hanau, Germany
CO ₂ Incubator CB150	Binder GmbH, Tuttlingen, Germany
Electrophoresis chamber	Bio-Rad Laboratories GmbH, Munich, Germany
Electrophoresis Unit MIDI PLUS (CRHU15)	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
Function line Incubator (bacterial culturing)	Heaeus Instruments, Hanau, Germany
GelDoc Go Gel Imaging System	Bio-Rad Laboratories GmbH, Munich, Germany
HeraCell150 (cell culture incubator)	Heaeus Instruments, Hanau, Germany
HeraSafe clean bench	Heaeus Instruments, Hanau, Germany

High-content microscope MetaExpressXL system	Molecular devices, Biberach, Germany
ImageXpress micro XL system (confocal microscope)	Molecular devices, Biberach, Germany
Incubator shaker C25	New Brunswick Scientific, Nürtingen, Germany
Innova 44 incubator shaker	New Brunswick Scientific, Nürtingen, Germany
Leica DM IL LED inverted laboratory microscope	Leica Microsystems GmbH, Wetzlar, Germany
Multichannel dispenser Explorer (300 µl, 1200 µl)	Eppendorf AG, Hamburg, Germany
NanoDrop2000c spectrophotometer	Thermo Fisher Scientific GmbH, Schwerte, Germany
NanoDrop1000 spectrophotometer	Thermo Fisher Scientific GmbH, Schwerte, Germany
Neubauer chamber	Brand, Wertheim, Germany
Octet RED96E system	Sartorius AG Göttingen, Germany
Overhead rotator	Bachofer, Reutlingen, Germany
PeqStar PCR cycler	VWR, Darmstadt, Germany
pH meter	Mettler-Toledo GmbH, Gießen, Germany
Pipettes 10 µl, 20 µl, 100 µl, 200 µl, 1000µl	Brand GmbH&Co KG, Wertheim, Germany
Pipetboy	Integra Biosciences, Fernwald, Germany
PowerPac BasicPower Supply	Bio-Rad Laboratories GmbH, Munich, Germany
Semi-dry electro blotters, PerfectBlue, Sedec	VWR International GmbH, Darmstadt, Germany
Sonifier Sonopulse HD60/UW60	Bandelin, Berlin, Germany
Sterile Hood	BDK GmbH, Sonnenbühl-Genkingen
ThermoMix500	SERVA Electrophoresis GmbH, Heidelberg, Germany
Thermomixer comfort	Eppendorf AG, Hamburg, Germany
Typhoon TRIO Scanner	GE Healthcare, Uppsala, Sweden
Vortex Genie 2	Scientific Industries, Karlsruhe, Germany
Water bath	Memmert, Schwabach, Germany

2.2 Molecular biology methods

2.2.1 Polymerase chain reaction

For amplification of DNA fragments, Polymerase chain reactions (PCRs) were performed using Q5 High-Fidelity DNA polymerase or Q5 high-fidelity 2x Master Mix (both New England Biolabs) with 10-20 ng template DNA according to the manufacturer's recommendations.

2.2.2 Agarose gel electrophoresis

DNA fragments were analysed and purified by agarose gel electrophoresis. For this, 6x DNA loading dye (New England Biolabs) was mixed with the sample and loaded onto a 1 % (w/v) agarose gel containing 1:50000 (v/v) Midori Green (NIPPON Genetics) in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1mM EDTA, pH 8.5). For DNA size determination, GeneRuler 1 kb plus DNA ladder (New England Biolabs) was used. DNA fragments were visualized with a GelDoc Go Gel Imaging System. Next, DNA fragments were cut out of the gel and purified using the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel) according to the manufacturer's instructions. DNA concentration was subsequently photometrically determined by NanoDrop (Thermo Fisher Scientific).

2.2.3 Restriction digest

Restriction digests of PCR products and plasmids were conducted with restriction enzymes from New England Biolabs according to the manufacturer's protocol. The desired fragments were subsequently purified by agarose gel electrophoresis as described above.

2.2.4 DNA Ligation

For DNA Ligation using T4 ligase (New England Biolabs), vector and insert were mixed in a 1:3 molar ratio using 100 ng vector. Ligations were performed either for 1 h at room temperature (RT) or overnight at 16 °C. Afterwards, the ligation was stopped by heat inactivation at 65 °C for 10 min.

2.2.5 Transformation of *E. coli*

For transformation of XL1, 50-100 ng ligation or plasmid were mixed with 25-50 µl chemical competent XL1 *E. coli* and incubated for 10-15 min on ice. After the heat shock for 40 s at 42 °C, bacteria were cooled for 2 min on ice. Then, 200 µl pre-warmed SOC media was added followed by incubation at 37 °C before plating on LB Agar plates with the respective selection antibiotic.

For transformation of large plasmids, NEB 10-beta competent *E. coli* (New England Biolabs) were used following the distributor's instructions.

2.2.6 Plasmid purification

Plasmids were extracted from *E. coli* using either the NucleoSpin Plasmid Mini Kit (Macherey-Nagel) for small-scale (5 ml culture) or the Qiagen Plasmid MidiPrep Plus Kit (Qiagen) for mid-scale (100 ml culture) purifications according to the manufacturer's instructions. DNA concentration was subsequently photometrically determined by NanoDrop (Thermo Fisher Scientific).

2.2.7 Cloning

To generate the mammalian expression constructs for the bivalent Drp1-Nbs, two DNA fragments encoding for the respective Nbs were connected by a flexible Gly-Ser linker [(G4S)₄] to which a C-terminal sortase tag (LPETG) followed by a His₆-tag were added. This construct was then inserted into a pCDNA3.4 expression vector variant comprising an N-terminal signal peptide for secretion³⁴¹. For bivD7, each Nb sequence was amplified separately by PCR using #1429 and #1430 or #1431 and #1133 primer pairs. Next, both Nbs were combined by fusion PCR using the primers #1289 and #1133. For bivD63 cloning, the primer pairs #1434 and #1244 as well as #1435 and #1133 were used followed by #1438 and #1133 for fusion PCR. Finally, the resulting DNA fragment was inserted into pCDNA3.4 by restriction digestion with Esp3I and EcoRI. For generation of unmodified Cbs, the Drp1-Nb sequences were inserted into pTagRFP via BglII and HindIII restriction digest as previously described³⁴². To generate turnover-accelerated Cbs, the Drp1-Nb sequence was cloned with PstI and BspEI into the previously described pEGFP-Ubi-R-3xGS-tagRFP plasmid²⁹⁵.

To generate the Dox-inducible vector, a tight TRE promoter including several tet operator elements as well as a custom multiple cloning site (MCS) and a polyA tail were inserted into pB-rtTA (a gift from Mauro Calabrese, Addgene #126034)³³⁸ by a two-step cloning protocol. In the first step, the TRE promoter was amplified from pSBet-GP (a gift from Eric Kowarz, Addgene #60495)³³⁹ using #2065 and #2066 primers. The PCR product was inserted into pB-rtTA via PacI and BglII restriction enzymes. In the second step, a DNA synthesis containing a MCS with NheI, SfiI, BstEII, XhoI, Bsu36I and Sall restriction sites as well as a poly A tail sequence (sequence taken from bivM114-Cb plasmid) was integrated via NheI and BglII into the vector generated in step1. For integration of eGFP into the Dox-inducible vector, eGFP was amplified from NM1603 using #2081 and #2082 primers and integrated via NheI-HF and Bsu36I.

For GBP-Fbox-TagRFP construct, each component was first amplified via PCR, then assembled by fusion PCR and subsequently cloned into the Dox-inducible vector. PCR1: Amplification of GBP (plus Kozak sequence) from GBP in pHEN6C and introduction of 5' SfiI restriction site using #2083 and #2084 primers. PCR2: Amplification of Fbox from pCDNA3_NSImb-vhhGFP4³⁰⁰ using #2085 and #2086 primers. PCR3: Amplification of GS-linker and TagRFP from bivD7 in pTagRFP plus introduction of 3' Sall-HF restriction site using #2087 and #2088 primers. PCR4: Fusion PCR of PCR1 and PCR2 with #2083 and #2086. PCR5: Fusion PCR of PCR3 and PCR4 using #2083 and #2088. The final PCR product was inserted into the Dox-inducible vector via SfiI and Sall-HF restriction enzymes. For exchange of Nb, SfiI and BstEII restriction sites were used.

For GBP-RBCC-TagRFP construct, each component was first amplified via PCR, then assembled via fusion PCR and subsequently cloned into the Dox-inducible vector. PCR1: Amplification of GBP (plus Kozak sequence) from GBP in pHEN6C and introduction of 5' XhoI restriction site using #2089 and #2084 primers. PCR2: Amplification of RBCC domain from HLTV-hTRIM21 (a gift from Leo James, Addgene #104973)³¹⁰ using #2090 and #2091 primers. PCR3: Amplification of GS-linker and TagRFP from bivD7 in pTagRFP plus introduction of 3' Sall restriction site using #2087 and #2088 primers. PCR4: Fusion PCR of PCR1 and PCR2 using #2089 and #2091. PCR5: Fusion PCR of PCR3 and PCR4 using #2089 and #2088. The final PCR product was inserted into the Dox-inducible vector via Sall-HF and XhoI restriction enzymes. For exchange of Nb, TRE promoter and the respective Nb were taken from the

respective Fbox-TagRFP construct and inserted via PacI and BstEII restriction enzymes.

BivM114 was generated by insertion of two M114-encoding sequences into the pCDNA3.4 expression vector: first, M114 sequence was amplified in two separate PCRs using primer sets #1373 + #1504 and #1374 + #1133. Next, both PCR products were then combined via fusion PCR using primers #1375 and #1263. The resulting DNA fragment was then cloned into Esp3I- and EcoRI- digested pCDNA3.4 expression vector by Gibson assembly according to the manufacturer's instructions.

For all constructs, the correct sequence was confirmed via Sanger sequencing. All expression constructs used in this study are listed in section 2.1.

2.3 Biochemical methods

2.3.1 SDS-PAGE and Western Blotting

For analysis with sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), protein samples were denatured (10 min, 95 °C) in 2x SDS-sample buffer (60 mM Tris/HCl, pH 6.8; 2 % (w/v) SDS; 5 % (v/v) 2-mercaptoethanol; 10 % (v/v) glycerol, 0.02 % (w/v) bromophenol blue). SDS-PAGE gels were prepared with a 12 % or 15 % separation gel (12 % or 15 % Acrylamide, respectively, 375 mM Tris pH 8.8, 0.1 % SDS, 0.05 % APS and 0.1% TEMED, all (v/v)) and a 6 % stacking gel (6 % Acrylamide, 60 mM Tris pH 6.8, 0.1 % SDS, 0.05 % APS and 0.1 % TEMED, all (v/v)). PageRuler plus prestained protein ladder (Thermo Fisher Scientific) was used for molecular weight determination. Proteins were separated with 200 V in SDS-PAGE running buffer (25 mM Tris, 192 mM Glycine, 0.1 % SDS (v/v), pH 8.3). Total protein was stained with Instant Blue staining solution (Abcam) for at least 15 min followed by washing off excess dye with ddH₂O.

For subsequent Western Blotting, proteins were transferred from the SDS-PAGE gels to a nitrocellulose membrane using a semi-dry blotting system with 150 mA per gel for 75 min (Nbs) or 105 min (Drp1). Successful transfer was confirmed by Ponceau S staining (0.1 % (w/v) Ponceau S, 5 % (v/v) acetic acid). Afterwards, membranes were incubated with the primary antibody (see section 2.1) in 5 % (w/v) bovine serum albumin (BSA) or 5 % (w/v) milk in TBST (50 mM Tris, 150 mM NaCl, 0.05 % (v/v) Tween20, pH 7.4) either 1 h at RT or overnight at 4 °C. Then, membranes were washed 3x 10 min with TBST and subsequently stained with secondary antibody (see section 2.1) in 5 % milk in TBST for 1 h at RT. Excess antibody was removed by additional 3x 10 min washing with TBST. The membrane was dried and imaged using a Typhoon-Trio laser scanning system (GE healthcare, excitation 633 nm and emission filter 670 nm BP 30 settings).

2.3.2 Expression and Purification of Nanobodies

Monovalent Drp1-Nbs and bivVB6^{253,285} were expressed and purified as described before^{243,269}. In brief, the encoding plasmid was transformed into XL1 *E. coli* and cultured in TB media at 37 °C. Nb expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD₆₀₀ 0.6-0.8 followed by overnight growth at 30 °C. Bacteria were harvested (6000 g, 10 min, 4 °C) and resuspended in IMAC binding

buffer (PBS, 500 mM NaCl, 50 mM Imidazole, pH 7.4). For bacterial lysis, DNaseI (1 µg/ml), phenylmethanesulfonylfluoride (PMSF, 1 mM), 1x Protease Inhibitor Mix B (Serva) and lysozyme (0.1 mg/ml) were added. Bacteria were sonicated for 8 min (10 s on, 10 s off), incubated for 1.5 h rotating at 4 °C and sonicated again. To remove cell debris, the lysate was centrifuged (20000 g, 30 min, 4 °C) and the supernatant filtered (0.45 µm filter).

Bivalent Drp1-Nbs and bivM114 were produced using the ExpiCHO system (Thermo Fischer Scientific) according to the manufacturer's recommendations^{335,341}. Then, the supernatant containing the secreted Nb was separated from the cells. For this, the cell suspension was first centrifuged with 500 g for 5 min and the supernatant subsequently centrifuged for additional 5 min by 7500 g. The resulting supernatant was filtrated (0.45 µm filter) and IMAC spike-in buffer added resulting in a final concentration of 50 mM Imidazole, 500 mM NaCl and 20 mM Na₂HPO₄.

Nbs were purified by immobilized metal ion affinity chromatography (IMAC) using the ÄKTA Pure 25 FPLC system (Cytiva) with a flow rate of 0.5-1 ml/min. 1 ml HisTrap FF columns (Cytiva) were equilibrated with 5 column volumes (CV) IMAC Binding buffer (PBS, 500 mM NaCl, 50 mM Imidazole, pH 7.4) or mammalian IMAC binding buffer (PBS, 500 mM NaCl, 20 mM Imidazole, pH 7.4) for purification from ExpiCHO cells. After sample application by sample pump, the column was washed with the respective binding buffer and the bound proteins were eluted by linear increase of the imidazole concentration to 500 mM. Afterwards, Nb-containing fractions and purity were determined by Coomassie Blue staining using InstantBlue solution (Abcam) and Western blotting against the His-tag (see section 2.3.1).

If the Nb purify was insufficient after IMAC, size exclusion chromatography (SEC) was performed for additional purification. SEC was performed using a Superdex 75 26/60pg gel filtration column with a flow rate of 2.6 ml/min. Depending on the downstream application, either PBS or Sortase buffer (50 mM Tris, pH 7.5, and 150 mM NaCl) were chosen. The column was equilibrated with 1.5 CV buffer. Starting from 0.2 CV after sample application, the elution was collected in 2 ml fractions. Nb purity was determined by Instant Blue staining.

Nanobody-containing fractions were concentrated by Amicon ultracentrifugation filters (Merck Millipore) according to the manufacturer's protocol. Protein concentrations were determined by spectrophotometer measurements.

2.3.3 Drp1 expression and purification

Drp1 was purified as described in¹⁰⁸ by A. Jenner. In brief, Drp1 isoform 1 was expressed from a pTYB2 vector in *E. coli* BL21-CodonPlus (DE3)-RIPL. *E. coli* were grown at 37 °C in LB medium with 100 µg/ml ampicillin and 35 µg/ml chloramphenicol. At OD_{600 nm} of 0.6, protein expression was induced using 1 mM IPTG and bacteria further grown at 14 °C for 18 h. Bacteria were harvested and cell pellets were resuspended in 20 mM HEPES/KOH pH 7.4, 500 mM NaCl and 1 mM MgCl₂ containing 1 mM PMSF, 1 µg/ml DNase I (Roche Diagnostics), and protease inhibitor (Complete; EDTA-free Protease Inhibitor Mixture; Roche Applied Science). After homogenization by mechanical rupture, cell debris was removed through centrifugation. Drp1 was purified using chitin resin affinity purification (New England Biolabs) and eluted by cleavage from the affinity resin using 30 mM DTT at pH 8 for 48 h at 4 °C. For further purification, anion exchange chromatography (HiTrap Q FF, GE Healthcare) was performed at pH 8. Pure elution fractions were combined, dialyzed in 20 mM HEPES/KOH pH 7.4, 500 mM NaCl and 1 mM MgCl₂ and stored with 20 % (v/v) glycerol. Purity was determined by SDS-PAGE and protein concentration was measured using Bradford assay and spectrophotometer measurements.

2.3.4 Affinity Measurements by biolayer interferometry (BLI)

Binding kinetics of Drp1-Nbs were determined using the Octet RED96e system (Sartorius) according to the manufacturer's instructions. For this, Drp1-Nbs were biotinylated by incubation with a 5x molar excess of Sulfo-NHS-LC-LC-Biotin in PBS for 30 min at RT. Unbound biotin was removed using Zeba Spin Desalting columns 7K MWCO 0.5 ml (Thermo Fisher Scientific) according to the manufacturer's recommendations. Then, streptavidin-coated biosensor tips (Sartorius) were incubated for 40 s with 1.7-10 µg/ml biotinylated Drp1-Nbs diluted in Octet buffer (HEPES, 0.1 % BSA). For association step, a 4-step 2-fold dilution series of Drp1 starting from 125 nM (D7), 62.5 nM (D63) or 120 nM (bivD7 and bivD63) were applied for 300 s which was followed by dissociation in Octet buffer for 720 s. For normalization, a reference applying Octet buffer only during association was used. Data analysis was performed with the Octet Data Analysis HT 12.0 software applying the 1:1 ligand model and global fitting.

2.3.5 Nanobody immobilization on NHS-Sepharose matrix

1.2 mg Drp1-Nb per 1 ml NHS-Sepharose (Cytiva) were immobilized following the manufacturer's recommendations.

2.3.6 Sortase labelling of nanobodies

For Sortase coupling, 50 μ M Nb, 250 μ M sortase peptide (H-Gly-Gly-Gly-propyl-azide, synthesized by AG Maurer, University of Tübingen) dissolved in Sortase buffer (50 mM Tris, pH 7.5, 150 mM NaCl) and 10 μ M Sortase were mixed in coupling buffer (sortase buffer with 10 mM CaCl_2) and subsequently incubated for 4 h at 4 °C. Uncoupled Nb and Sortase were removed by IMAC, and the excess peptide was depleted via Amicon Ultra Centrifugal Filters (Merck Millipore). For labelling of the azide-coupled Nb with a fluorophore, SPACC (strain-promoted azide-alkyne cycloaddition) click chemistry reaction was applied. For this, 5x molar excess of dibenzocyclooctyne-AlexaFluor647 (DBCO-AF647) (Jena Bioscience) was mixed with the azide-coupled Nb followed by incubation for 2 h at RT. The AF647-coupled Nbs was purified by dialysis and subsequent hydrophobic interaction chromatography (HIC).

BivVB6 and bivBC2 functionalization was performed analogously using a slightly different substrate peptide (H-Gly-Gly-Gly-Arg-Arg-Lys(N_3)- NH_2 , synthesized by AG Schwarzer, University of Tübingen) and dibenzocyclooctyne-AlexaFluor488 (DBCO-AF488) (Jena Bioscience). The HIC purification step was omitted.

For generation of the bivM114-trap, 1 mg of azide-coupled bivM114 was incubated with 0.5 ml DBCO-Agarose slurry (Jena Bioscience) for 4 h at 25 °C. To confirm successful reaction of the Nb, input and supernatant after reaction were analysed by SDS-PAGE. The Nb-coupled beads were washed thrice with 2.5 ml PBS (2700 g for 2 min centrifugation steps) and stored in PBS with 0.05 % (w/v) sodium azide.

2.3.7 Mammalian cell lysis and protein extraction

For intracellular immunoprecipitation (IC-IP), $2.5 - 3 \times 10^6$ HEK293 cells were seeded in a 100 mm culture dish (P100). The next day, cells were transfected with the Cb-encoding plasmid. 24 h after transfection, cells were harvested at ~90 % confluency. For immunoprecipitations (IPs) using Nb-traps, T175 flasks or P100 dishes of HEK293, U2OS or HeLa cells were harvested at ~90 % confluency. HEK293 cells were detached

by light pipetting, U2OS and HeLa cells by trypsinization. Afterwards, the cells were washed once with 1 ml PBS, and the cell pellets were stored at -80 °C until lysis. For lysis, T175 flask or P100 pellets were resuspended in 600 µL or 200 µL lysis buffer (50 mM Tris HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8; 0.5 % (v/v) TritonX-100; 1 µg/µL DNaseI; 2.5 mM MgCl₂; 2 mM PMSF; 1x Protease inhibitor Mix M (Serva)), respectively. For mechanical cell rupture, the lysate was passed through 20G, 23G and 27G needle gauges multiple times. Between the needle steps, the lysate was incubated for 10 min on ice, vortexed, and incubated for additional 10 min. Subsequently, the lysate was centrifuged (10 min, 18000 g, 4 °C), the supernatant transferred to a fresh tube and diluted 1.5-fold with dilution buffer (50 mM Tris HCl pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8; 2 mM PMSF).

2.3.8 Immunoprecipitation

Soluble protein extracts were generated from HEK293, HeLa and U2OS cells as described in section 2.3.7. For preparation of the input sample (IN), 20 µL of the lysate were mixed with 20 µL 4x SDS-Sample buffer (see above). For IP, 80 µL slurry of each Nb-trap was equilibrated in IP buffer (50 mM Tris HCl pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8) and added to equal amounts of lysate. IC-IP experiments were performed with 80 µL of RFP-trap slurry (ChromoTek). Soluble protein extracts were incubated with the Nb-traps overnight at 4 °C on an end-over-end rotor. Alternatively, for positive control (precipitation of endogenous Drp1), 4 µL anti-Drp1 antibody was added to the lysate and following overnight incubation at 4 °C, 80 µL equilibrated Protein A/G Sepharose slurry were added and incubated for additional 3-4 h at 4 °C. Beads were retrieved by centrifugation (2000 g, 2 min, 4 °C) and supernatant (non-bound sample, NB) was mixed 1:1 with 4x SDS-sample buffer. The beads were washed thrice with 500 µL wash buffer (IP buffer with 2 mM PMSF, for MS samples additionally 1x Protease inhibitor mix and 0.05 % (v/v) Tween20). During the third wash, beads were transferred into a fresh tube. The beads were boiled in 60 µL 2x SDS-sample buffer for 10 min at 95 °C and the supernatant was transferred into a fresh tube (bound sample, B).

For immunoblotting of IP samples, 1 % of input and non-bound and 33 % of the bound sample were loaded on an SDS-PAGE gel and transferred to nitrocellulose membrane as described in section 2.3.1. Immunoblots were probed with the following antibodies:

anti-Drp1, anti-TagRFP, anti-GAPDH. All antibodies used in this study are listed in section 2.1. Immunoblots were scanned using a Typhoon-Trio laser scanner (GE Healthcare) with excitation 633 nm and emission filter 670 nm BP 30 settings.

2.3.9 Liquid chromatography-MS sample preparation and measurement

Immunoprecipitations of Drp1- or Miro1-traps were compared to the GFP-trap (negative control) in three technical replicates. Immunoprecipitation was performed as described in section 2.3.7, proteins separated by SDS-PAGE (4-12% NuPAGE tris gel (Invitrogen)) for 7 min at 200 V and subsequently stained with Coomassie blue. The protein gel pieces were digested by trypsin as previously described³⁴³. Purified peptide samples were measured on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) online-coupled to an Easy-nLC 1200 UHPLC (Thermo Fisher Scientific). Peptides were separated using a 20-cm-long, 75- μ m-inner diameter analytical HPLC column (ID PicoTip fused silica emitter; New Objective, Berks, UK) packed in-house with ReproSil-Pur C18-AQ 1.9- μ m silica beads (Dr Maisch GmbH) and eluted using a 60 min segmented gradient from 10 - 90 % of solvent A (0.1 % formic acid) and solvent B (80 % acetonitrile in 0.1 % formic acid) at a constant flow rate of 200 nl/min. The column temperature was maintained at 40 °C by an integrated column oven. Peptides were ionized by nanospray ionization and a capillary temperature of 275 °C. The mass spectrometer was operated in the positive ion mode. Full MS scans were acquired in a range of 300-1750 m/z at resolution of 60,000 and seven most intense multiple-charged ions were selected for HCD fragmentation with a dynamic exclusion period of 30 s and tandem MS (MS/MS) spectra were acquired at resolution of 15,000.

2.4 Cell culture methods

2.4.1 Culturing of mammalian cells

The HeLa Kyoto cell line (Cellosaurus no. CVCL_1922) was acquired from S. Narumiya (Kyoto University, Japan). Drp1 KO HeLa cells were generated in and provided by the lab of Thomas Langer (Max Planck Institute, Cologne, Germany), HeLa_{BC2T}*ACTB* cells were generated during a previous study in our lab²⁸⁵. The U2OS (HTB-96) and HEK293 (CRL3216) cell lines were acquired from ATCC. The mycoplasma PCR Detection kit (abm) or the mycoplasma kit Venor GeM Classic (Minerva Biolabs) and Taq polymerase (Minerva Biolabs) were applied for mycoplasma testing. As this study does not contain cell-line specific experiments, no additional authentication was performed. Cell culturing was conducted using standard protocols. In brief, HeLa, HEK293 and U2OS cells were cultured in DMEM high glucose, pyruvate, GlutaMax with 10 % (v/v) fetal calf serum and 1 % (v/v) penicillin/streptomycin (all Thermo Fischer Scientific). A549 cells were cultured in DMEM/F12 (high glucose, high pyruvate) with 10 % (v/v) fetal calf serum, 1x GlutaMax and 1x penicillin/streptomycin (all Thermo Fischer Scientific). For routine passaging of cells, 0.05 % trypsin-EDTA (Thermo Fischer Scientific) was used. Cells were cultivated at 37 °C and 5 % (v/v) CO₂ in a humidified incubator.

2.4.2 Transfection

U2OS cells were transiently transfected with Lipotectamine2000 (Thermo Fisher Scientific) according to the manufacturer's protocol. HEK293 and HeLa cells were transfected using Polyethyleneimine (PEI, Sigma Aldrich) as previously described²⁷².

2.4.3 Cell cultivation, seeding, and staining for super resolution microscopy

U2OS wild type or U2OS mEGFP-Drp1 cells were cultivated in DMEM (low glucose), 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin (Invitrogen) at 37 °C and 5 % (v/v) CO₂. Prior to microscopy, cells were seeded in 35 mm μ -Dish 1.5H glass bottom imaging chambers (for confocal imaging) or on 35 mm 1.5H glass coverslips in a 6-well plate (for SMLM) at a density of 3×10^5 cells per dish/well and grown for 18 h. If applicable, mitochondria were stained with 200 nM MitoTracker Green FM (Invitrogen) for 20 min. Subsequently, cells were fixed in 4 % (v/v) PFA in DMEM (pre-warmed to

37 °C) for 10 min at RT and washed twice with PBS. To quench unreacted fixative, cells were incubated in 50 mM NH₄Cl for 15 min followed by permeabilization with 0.25 % (v/v) Triton X-100 for 8 min. Next, cells were washed with PBS thrice for 5 min and unspecific binding sites were blocked with 1 % (w/v) BSA in PBS for 1 h. Drp1 was labelled using 250 nM bivD7 coupled to AlexaFluor 647 (bivD7_{AF647}) in 1 % (w/v) BSA in PBS overnight at 4 °C in the dark. The cells were washed thrice with PBS and stored protected from light at 4 °C until imaging.

2.4.4 Culturing and transfection of ExpiCHO cells

For ExpiCHO cells, the required media and transfection reagents were obtained from Thermo Fisher Scientific. Culturing and transfection were performed according to the manufacturer's recommendations.

2.4.5 Cho-coo protein transduction

Cho-coo stock preparation

Cholesterol-coomassie (cho-coo) was kindly provided by Xiaohu Gao (University of Washington, USA). The dried cho-coo was reconstituted in a 4:1 (v/v) ethanol/water mixture to a 1.6 mM final concentration and stored at 4 °C. Before use, the stock was warmed to RT and quickly incubated in an ultrasonic bath to resolve any precipitates.

General cho-coo transduction protocol

The Nb_{AF488} and cho-coo were diluted in PBS to 10 % of the final volume. Next, the mixture was incubated for 8 min at 37 °C in a water bath, followed by 1-2 min in an ultrasonic bath and additional 8 min at 37 °C. Then, the mixture was filled up with OptiMEM and Hoechst 33258 (1 µg/ml final concentration). Cells were washed thrice with OptiMEM and 90 µl transduction mix was added followed by incubation for 1 h at 37 °C, 5 % CO₂ in a humidified incubator. Next, cells were washed thrice with OptiMEM and 100 µl imaging medium (Fluorobright media + 10 % (v/v) FBS + 1x GlutaMax + 1x Penicillin/ Streptomycin, all Thermo Fisher Scientific) was added. Cells were imaged using MetaExpressXL high-content microscope. Microscopy images were processed using the MetaExpress software (64 bit, 6.2.3.733 or higher, Molecular Devices).

Initial testing using bivBC2-AF488

Wild-type (wt) HeLa cells and BC2T*ACTB* HeLa cells²⁸⁵ were seeded at 10000 cells/well. The next day, cells were transduced using 100 nM bivBC2_{AF488} and a 1:10 molar cho-coo ratio.

Ratio testing

HeLa and U2OS cells were seeded with 8000 cells/well. For transduction, 100 nM bivVB6_{AF488} was used in a 1:8, 1:12, 1:16 or 1:20 ratio with cho-coo. A cho-coo only sample was prepared as well.

Seeding densities

To test for optimal confluency at transduction, 3000, 6000 or 9000 cells/well of HeLa, U2OS or A549 cells were seeded. For transduction, 100 nM bivVB6_{AF488} in a 1:8 or 1:16 Nb_{AF488} to cho-coo ratio was used.

Time lapse experiment

For time lapse microscopy, HeLa and A549 cells were seeded at 3000 cells/well seeding density and transduced using a 1:8 ratio with 100 nM fluorescently labelled Nb. Cells were imaged for 0 h, 6 h, 24 h and 48 h.

2.5 Microscopy

2.5.1 Chromobody imaging

For Chromobody imaging, wt HeLa cells (8000 cells/well) or Drp1 KO HeLa cells (11000 cells/well) were seeded in μ clear 96 well plates (Greiner Bio-One). The next day, cells were transfected with the respective plasmids. 24 h post transfection, cells were stained with 400 nM MitoTracker green (Life Technologies) and 4 μ g/ml Hoechst33342 (Thermo Fisher Scientific) for 30 min. Afterwards, the medium was replaced with imaging medium (DMEMGfp-2 (Evrogen) supplemented with 10 % (v/v) FBS and 2 mM L-glutamine) with or without 20 μ M Carbonyl cyanide m-chlorophenylhydrazone (CCCP). Images were taken 10 min, 70 min and 130 min after CCCP addition with an ImageExpress Micro Confocal High Content screening system (Molecular devices) at 40x magnification. Cells were kept at 37 °C and 5 % CO₂ during imaging. Raw microscopy images were adjusted in brightness and contrast using MetaExpress software (64 bit, 6.2.3.733 or higher, Molecular devices).

2.5.2 Immunofluorescence in fixed cells

Cells were seeded in μ clear 96 well plates (Greiner Bio-One) as described in section 2.5.1. The next day, cells were transfected with GFP-Drp1 plasmid or left untransfected. 24 h after transfection, cells were washed twice with PBS and subsequently fixed with 3.7 % paraformaldehyde (PFA) for 10 min at RT. After washing with PBS thrice, cells were incubated with TBST for 10 min at RT. Following three additional washes, cells were blocked with 5 % BSA in TBST for one hour. Next, cells were incubated with 100-1000 nM Nb and anti-Drp1 Ab (1:100-1:300) in 5 % BSA/TBST overnight at 4 °C. Unbound Nb and Ab were washed away followed by incubation with secondary Abs (anti-rabbit labelled with AF488 (1:1000) and Cy5-conjugated goat anti-alpaca Ab (1:500)) for 1 h at RT in 2.5 % BSA/TBST. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) simultaneously. After additional washes with TBST and PBS, images were taken using an ImageExpress Micro Confocal High Content screening system (Molecular devices) at 40x magnification. Raw microscopy images were adjusted in brightness and contrast using MetaExpress software (64 bit, 6.2.3.733 or higher, Molecular devices).

2.5.3 Confocal fluorescence microscopy

U2OS mEGFP-Drp1 cells were prepared as described in section 2.4.3. Confocal fluorescence microscopy images were acquired on an inverted Infinity Line confocal dual-colour STED 775 QUAD laser scanning microscope (Abberior Instruments) equipped with a UPLXAPO 60x/1.42 Oil objective (Olympus). mEGFP and AF647 were excited at 488 nm and at 640 nm wavelength, respectively. Images were acquired with a pixel size of 65 nm and a pixel dwell-time of 5 μ s. The fluorescence emission signal was collected on avalanche photodiode detectors. Raw microscopy images were adjusted in brightness and contrast using Fiji/ImageJ³⁴⁴. Colocalization of endogenous mEGFP-Drp1 and bivD7_{AF647} was assessed by Pearson's correlation coefficient calculation of the respective emission signals using Just Another Co-localization Plugin (JACoP)³⁴⁵ in Fiji/ImageJ³⁴⁴. For this, the images were cropped to exclude unspecific bivD7_{AF647} signal outside of the cell and in the nucleus. Experiments are representative for n = 3 individual replicates with n = 11 cells in total.

2.5.4 Single-molecule localization microscopy

U2OS wild type cells were seeded and stained as described in sections 2.4.3 and 2.5.3. Next, cells were mounted on custom-made imaging chambers or single concave depression microscopy slides covered with 100-300 μ L imaging buffer (50 mM Tris/HCl pH 8, 10 mM NaCl, 10 % (w/v) glucose, 35 mM cysteamine (MEA), 0.5 mg/ml glucose oxidase (Sigma) and 40 μ g/ml catalase (Sigma)) and sealed using Twinsil speed dental glue (Picodent, Germany). Cells were imaged on a home-built wide-field/TIRF microscope or on a SR GSD (3D) inverted DMI6000 B super-resolution wide-field/TIRF microscope (Leica Microsystems) equipped with a 100x, 1.47 N.A. GSD Objective, 405 nm, 488 nm, 532 nm and 642 nm excitation lasers and an Andor iXon Ultra 897 EMCCD camera. Image series were acquired with an 30-50 ms exposure time and an EM gain of 300. AF647 was excited using 640 nm wavelength and the 405 nm activation laser intensity was manually adjusted to maintain a constant number of localizations per frame. Typically, 70,000–100,000 frames were recorded. Analysis was performed using the LAS X Software (Leica) and ThunderStorm³⁴⁶. Images were rendered using a Gaussian with a width according to the localization precision. For image analysis, Fiji/ImageJ³⁴⁴ was used. Images are representative for n = 6 individual experiments.

2.5.5 Testing of Dox-inducible plasmid

6000 cells/well wt HeLa cells were seeded into a µclear 96 well plate (Greiner Bio-One). The next day, cells were transfected with 100 ng/well GFP-Dox-inducible-vector plasmid using Lipofectamine2000 (Thermo Fisher Scientific). 24 h post transfection, cells were treated with 0, 10, 100 or 1000 ng/ml Doxycycline (Sigma-Aldrich) in imaging media (Fluorobright media +10 % (v/v) FBS +1x GlutaMax + 1xPenicillin/Streptomycin, all Thermo Fisher Scientific) and imaged 0 h, 4 h, 6 h, 8 h, 24 h and 48 h post induction using a MetaExpressXL high-content microscope.

2.5.6 Testing of Nb-degrons

For testing the degradation of GFP-Drp1, wt HeLa cells were seeded 10000 cells/ well in a µclear 96 well plate. After 24 h, cells were transfected in an equimolar ratio of a degron plasmid and a GFP-Drp1 encoding plasmid using Lipofectamine2000 (100 ng/well total DNA). 24 h post transfection, cells were stained with Hoechst33342 (1 µg/ml, Thermo Fisher Scientific) for 30 min, washed once and degron expression induced by addition of 1 µg/ml Doxycycline (Sigma-Aldrich) in imaging media. Cells were subsequently imaged 0 h, 8 h, 24 h and 30 h after Doxycycline addition.

For testing degradation of endogenous Drp1, wt HeLa cells were seeded at 9000 cells/well and Drp1 KO HeLa cells at 10000-15000 cells/well. The next day, wt HeLa cells were transfected with 100 ng/well degron plasmid using Lipofectamine2000 while Drp1 KO HeLa cells were left untransfected. Before starting Doxycycline treatment, cells were cultivated for 24 h post transfection to allow plasmid uptake. Cells were treated for 0 h, 8 h, 24 h, and 30 h with 1 µg/ml Doxycycline in HeLa culture medium (see section 2.4.1). Afterwards, cells were stained with 1 µg/ml Hoechst33342 and 400 nM MitoTracker green (Cell Signaling Technology) for 30 min, washed once and imaged in imaging media. For Drp1 Ab detection, cells were subsequently fixed with 3.7 % PFA for 10 min at RT. Cells were washed three times with PBS and incubated with TBST for additional 10 min at RT. Afterwards, cells were washed again, blocked using 5 % BSA in TBST for 1 h at RT and stained overnight with 1:100 anti-Drp1 Ab. Next, cells were washed three times with PBS and stained with secondary Ab (AF647-labelled anti-rabbit Ab, 1:1000) and 1 µg/ml Hoechst33342 for 1 h at RT. Cells were subsequently washed with TBST and PBS and imaged using a MetaExpressXL high-content microscope.

2.6 Data analysis, Statistics and Software

2.6.1 Mass spectrometry data analysis

The acquired raw files of MS data were processed by MaxQuant software (version 2.0.3.0.)³⁴⁷ and searched against the Uniprot *Homo sapiens* database (105,079 entries, downloaded 2022/08/01), *Vicugna pacos* derived nanotraps and commonly observed protein contaminants. For MS and MS/MS, the peptide mass tolerance was set at 4.5 ppm and 20 ppm, respectively. Only two missed cleavages were allowed for the tryptic digestion. Carbamidomethylation (C) was used as fixed modification, while oxidation (M) and acetylation (protein N-term) were defined as variable modifications. False discovery rate was set to 1 % for both peptide and protein level. For label-free quantification a minimum ratio count of two was requested. Intensity-based absolute quantification (iBAQ) was enabled. Downstream analysis of the 'proteinGroups.txt' output table was performed using Perseus (version 1.6.15.0). Contaminants, reversed proteins and proteins only identified by site were excluded and only proteins that were quantified in two out of three replicates of either bait or control pulldown were retained in the dataset. Missing values were imputed (width 0.3, down shift 1.8). To determine significantly enriched proteins, a multi-volcano analysis (Hawaiian plot) was performed. The s_0 and FDR parameters of the multi-volcano analysis were: for class A (higher confidence, $s_0 = 0.1$, FDR = 0.01) and class B (lower confidence, $s_0 = 0.1$, FDR = 0.05). The R environment (version 4.1.1) and GraphPad (version 8.0.1.) were used for additional graphical visualization.

The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE³⁴⁸ partner repository with the dataset identifier PXD051737.

2.6.2 Image analysis and statistics for chromobodies

Image analysis of Cb data was performed with MetaExpress software (64 bit, 6.2.3.733 or higher, Molecular devices) for $n > 130$ cells per construct. Using the Custom editor (version 2.5.13.3 or higher) of the MetaExpress software, an algorithm was developed to determine the average signal intensity per transfected cell. Based on the assumption that cells usually are transfected with both plasmids, the GFP-NLS signal was used as a marker for transfection and cell area. Cell average intensity of the TagRFP signal was determined for all GFP-positive cells. To quantify the background signal, the TagRFP signal in GFP-NLS negative cells ($n = 2125$ cells) was measured using the

DAPI signal for cell localization and subtracted from all measurements. A workflow is shown in **Appendix I: Figure S14**³³⁶.

Mean and standard deviation were calculated for each condition. For comparison of unmodified and turnover-accelerated Drp1-Cbs, significance was calculated by Kruskal Wallis test with Dunn's multiple comparisons test. To compare turnover-accelerated Cbs in wt HeLa and Drp1 KO HeLa cells, significance was calculated using two-way ANOVA with Sidak's multiple comparisons test. Statistical tests and graph preparation were done using GraphPad Prism software (Version 8 or higher).

2.6.3 Image analysis and statistics for Drp1-degrons

Image analyses were performed using the MetaExpress software (64 bit, 6.2.3.733 or higher) from Molecular devices.

GFP intensity in TagRFP positive cells

The "Find round objects" module was used to identify TagRFP positive cells (min. width 15 µm, max. width 80 µm, intensity above local background 200). The GFP signal intensity was then measured in the identified cells.

GFP intensity in all GFP-Drp1 positive cells

To measure the overall signal of GFP-Drp1 positive cells, first the nuclei were identified using a "Find round objects" module (min. width 5 µm, max. width 30 µm, intensity above local background 200). Next, "Grow objects without touching" by 10 pixels was used to expand "Find round objects" to roughly fit the whole cell. Then, a "Filter Mask" was applied to exclude non-transfected cells using a min. filter with minimum value of 300 of GFP signal (measurement in total area of "Grow objects without touching"). Finally, the GFP-Drp1 signal was measured in all cells not excluded by the "Filter Mask".

Drp1 signal in fixed cells

To determine the signal of Drp1 stained with anti-Drp1 Ab and AF488-labelled secondary Ab, first the nuclei were identified using a "Find round objects" module (min. width 5 µm, max. width 30 µm, intensity above local background 200). Next, "Grow

objects without touching” by 10 pixels was used to expand “Find round objects” to roughly fit the whole cell and the AF488-signal was measured.

The GFP intensity in TagRFP positive cells was normalized to GFP intensity in all GFP-Drp1 positive cells (GFP-Drp1 transfected cells) or Drp1 signal in fixed cells (endogenous Drp1). Data are presented as median +95 %CI. For Fbox n>1268 cells over 4 biological replicates, for RBCC n>539 cells over 3 biological replicates, and for endogenous Drp1 n>1735 cells over 2 biological replicates were quantified. Significance was calculated using Kruskal Wallis test with Dunn’s multiple comparisons test. GraphPad Prism software (Version 8 or higher) was used for statistical tests and graph preparation.

2.6.4 Quantification of cho-coo experiments

To count the overall cells, first “count nuclei objects” (min width 5 µm, max width 40 µm, intensity above local background 200) was applied followed by “grow objects without touching” grown by 25 pixels. Identified cells were counted. To count positive cells, an additional “Filter Mask” measuring the average intensity with a minimum value of 4500 was applied to the AF488-signal and the identified cells counted. Percentage of positive cells in the overall population was determined, and graphs were prepared using GraphPad Prism software (Version 8 or higher).

2.6.5 Structure Prediction using AlphaFold

The structure predictions of Drp1 isoform1 and Miro1 isoform1 were taken from the AlphaFold Protein Structure Database with a model creation date of 1 Aug 2025^{72,73}. Colouring according to model confidence (dark blue=very high (pLDDT>90), light blue=high (pLDDT >70), yellow=low (pLDDT >50), orange=very low (pLDDT<50)).

The structure of D7 Nb depicted in Figures 7 and 8 was generated 18 Feb 2026 using AlphaFold3 via the AlphaFold Server (<https://alphafoldserver.com>)²⁵¹. As input, D7 amino acid sequence as depicted in Appendix I: Supplementary Table 1 was used. Colouring according to model confidence (dark blue=very high (pLDDT>90), light blue=high (pLDDT >70)).

2.6.6 Network visualization with Cytoscape

Cytoscape Web 1.0 (<https://cytoscape.org>)^{349,350} was used to visualize the significantly enriched proteins identified by bivM114-trap (creation date 20 Feb 2026). Darker colour indicates higher $-\log_{10}(\text{p-value})$.

2.6.7 Interaction visualization by STRING database

Visualization of Miro1 first shell of interaction (Figure 6) was taken from the STRING database (<https://string-db.org>)^{183,184} on the date of 20 Feb 2026.

2.6.8 Correction of Spelling and Grammar using Gemini

Correction of spelling and grammar by artificial intelligence as additional aid was permitted by the examiners (see document above). For this, Gemini 1.5 Pro (Google) versions March 2026-April 2026 was used. The prompts covered the following parameters: correct spelling and grammar according to British English, explicitly no corrections due to style, highlight made changes, give explanations for changes of grammar, give no explanations for already correct grammar, check if correction and original are identical before output.

3 Results

3.1 Results Drp1

Parts of this chapter were previously published in *Life Science Alliance* under the terms of the Creative Commons License Attribution 4.0 International (published 17.7.2024)³³⁶:

Nanobodies as novel tools to monitor the mitochondrial fission factor Drp1

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Author contributions:

Author	Author position	Scientific ideas %	Data Generation %	Analysis & Interpretation %	Paper Writing %
Theresa Fröhlich	1	50	50	25	70
Title of paper:		Nanobodies as novel tools to monitor the mitochondrial fission factor Drp1			
Status in publication process:		published			

I (T. Fröhlich), F. Fagbadebo and U. Rothbauer designed the study. S. Nueske and A. Scholz immunized the alpaca with Drp1 provided by A. Jenner. The two Drp1 specific Nb lead candidates (D7, D63) were initially selected by F. Fagbadebo. Further characterization (BLI measurements, domain mapping, initial immunoprecipitations, initial fluorescence imaging, cloning of all Cb constructs, initial Cb testing (including IC-IP shown in Figure 14B)) was done by F. Fagbadebo (see also³³⁵). For context, these

initial results my work is built upon are summarised in section “Generation, characterisation and initial application of Drp1 Nbs”. I performed an additional in-depth characterization of both candidates and reproduced data for the monovalent versions. I designed, cloned, produced and functionalized the bivalent version of the lead candidates (bivD7, bivD63) which I further characterized by affinity measurements (BLI, with support of D. Frecot). I performed all shown immunoprecipitations (and immunoblotting) while subsequent MS, MS data analysis and graph preparation (Figure 11) were performed by C. Cavarischia-Rega. I performed all shown confocal microscopy including sample preparation and statistical analysis of both fixed cells and live cell Cb imaging. A. Jenner performed STORM imaging (plus the confocal imaging shown in Figure 13), accompanying image analysis and prepared Figure 13. Y. Lurz performed GTPase assays. E. Schäffer, A. Garcia-Saez, B. Maček and U. Rothbauer supervised this study. I and U. Rothbauer drafted the article. All authors edited the article.

Data of this published manuscript was also included in F. Fagbadebo’s dissertation submitted to the Faculty of Science at the University of Tübingen in 2022³³⁵.

The sections “Generation of Dox-inducible vector and degron design”, “Testing Fbox-based Drp1-degrons”, “Testing RBCC-based Drp1-degrons” and “Degradation of endogenous Drp1” were not previously published.

Generation, characterisation and initial application of Drp1 Nbs

To generate Drp1-specific Nbs, an alpaca (*Vicugna pacos*) was immunized with recombinant Drp1. After confirmation of a specific immune response by serum ELISA, PBMCs were isolated from blood of this alpaca. From these PBMCs, mRNA was extracted and reverse transcribed into cDNA, which served as template for amplification of the VHH-encoding sequences by PCR. The resulting amplicons were cloned into a phagemid vector thus establishing an anti-Drp1 VHH phagemid library. This library was subsequently screened for Drp1-specific binding molecules by phage display, and the eight unique phase ELISA-positive clones identified were further tested by protein ELISA. Hereby, two diverse Drp1-Nbs, D7 and D63, showed strong signals and thus were selected for further studies (**Appendix I: Figure S1**^{335,336}). Then, both Nbs were expressed in *E. coli* and purified by IMAC and subsequent SEC (**Appendix I: Figure 1A,B**^{335,336}). Both D7 and D63 showed high affinities in the low nanomolar range with K_D s of 3.5 nM and 1.8 nM, respectively, as measured by BLI (**Appendix I: Figure 1C, and S2**^{335,336}). To test their suitability for capturing endogenous Drp1, both Nbs were covalently coupled to NHS-activated Sepharose beads to generate so-called Drp1-traps, which indeed precipitated endogenous Drp1 from HEK293 cell lysate³³⁵. Next, these Drp1-traps were used to investigate which Drp1 domains are bound by the Nbs. For this, different Drp1 deletion constructs were transiently expressed in HEK293 cells, and it was examined if they were precipitated by the Drp1-traps. This showed that D7 exclusively recognizes the N-terminal GTPase domain while D63 requires the GED for binding (**Appendix I: Figure 2**^{335,336}).

In addition, both D7 and D63 were tested as primary binding molecules in immunofluorescence imaging. Here, initial experiments indicated that none of the Nbs stained exogenously expressed GFP-Drp1 or endogenous Drp1 in fixed cells³³⁵.

To visualize Drp1 in live cells, mammalian expression vectors were generated that contain Nb-encoding sequences genetically fused to TagRFP, so-called “Chromobodies” (Cbs). First, their suitability for cytoplasmic expression was determined by intracellular immunoprecipitation (IC-IP). For this, Cbs were precipitated from the soluble protein fractions of HEK293 cells transiently expressing the Cbs using an RFP-trap, and the bound fractions were analysed for enrichment of Drp1. After precipitation of D7-Cb and D63-Cb, a substantial enrichment of Drp1 was detected in the bound fractions, which was not seen for a non-Drp1-specific Cb used as a negative control³³⁵. However, preliminary live-cell imaging did not reveal a Drp1-typical

distribution of the Cb signal. Consequently, both Drp1-Cbs were considered to be unsuitable for live-cell imaging of Drp1 at this stage³³⁵.

Application of Drp1-traps to capture endogenous Drp1 and analysis of the precipitated Drp1 interactome via mass spectrometry

After the initial demonstration that the Drp1-traps were able to pulldown endogenous Drp1 from HEK293 cell lysate³³⁵, we asked whether the Drp1-traps could also be used to study the Drp1 interactome. First, I tested if the Drp1-traps were able to precipitate endogenous Drp1 from other cell lines as well. For this, soluble protein extracts from three human cell lines (HEK293, HeLa, and U2OS) were generated and subsequently incubated with the Drp1-traps. As controls, a monoclonal anti-Drp1 Ab immobilized on protein A/G-Sepharose beads (positive control (PC)) and the GFP-trap²⁶⁹ (negative control (NC)) were used. Immunoblot analysis revealed comparable pulldown efficiencies for Drp1 with both Drp1-traps for all cell lines tested (**Figure 10**). Of note, both Drp1 traps precipitated Drp1 more efficiently than the anti-Drp1 Ab (**Figure 10**).

As immunoprecipitations are performed under non-denaturing conditions, not only the POI will be enriched but interacting proteins will be co-immunoprecipitated allowing further insights into the POI's function and regulation. Therefore, we analysed the interactome of both Drp1-traps by MS (in collaboration with C. Cavarischia-Rega, AG Mačák). For this, immunoprecipitations were performed in triplicate for each cell line, with each replicate containing the same number of cells as starting material. As exemplary shown for HEK293 cells, the replicates were highly comparable, showing Spearman rank correlations close to one (**Figure 11A**). As expected, the correlation between the two Drp1-traps was higher compared to each trap and the negative control (GFP-trap), as shown by principal component analysis (PCA) (**Appendix I: Figure S4B**³³⁶). In addition, the number of identified protein groups (PGs) was comparable between all samples showing a background of up to 981 proteins.

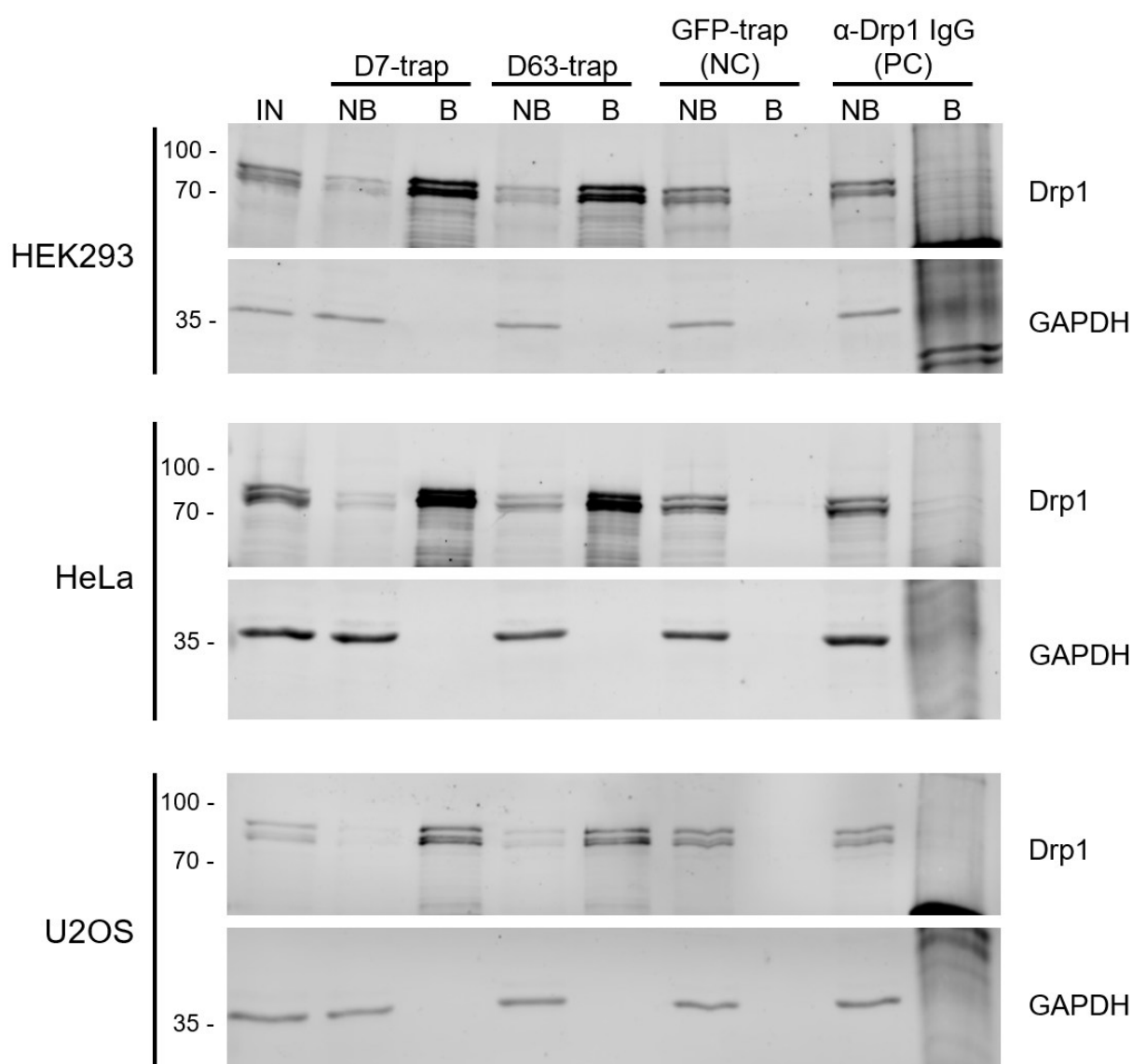


Figure 10: Validation of Drp1-traps by immunoprecipitation

For immunoprecipitation, the soluble protein fractions of HEK293, HeLa or U2OS cells were incubated with Drp1-traps (D7 and D63-trap), GFP-trap (non-specific trap, negative control (NC)) or anti-Drp1 Ab immobilized on Protein A/G beads (positive control (PC)). Input (IN, 1% of total), non-bound (NB, 1% of total) and bound (B, 33% of total) fractions were subjected to SDS-PAGE following immunoblotting with anti-Drp1 (upper panels) or anti-GAPDH (lower panels) Ab. Molecular weight in kDa is indicated on the left. Figure adapted from³³⁶.

Then, the capturing of Drp1 by the traps was analysed. With both D7- and D63-traps numerous Drp1 peptides were detected, resulting in a high coverage (~70 %) of the Drp1 sequence (**Figure 11B**). Notably, 55 "Razor" peptides matching Drp1 isoform 1, 5, 6 or 7 were identified in the precipitates of both Drp1-traps (**Figure 11B**). However,

this dataset does not allow us to differentiate between these isoforms. For both Drp1-traps, Drp1 was among the of the most abundant proteins identified, while being not detected in the NC (**Figure 11C**). In addition, Drp1 was the most significantly enriched protein for both Drp1-traps (**Figure 11D**). Finally, the MS data was analysed for potential Drp1 interactors. Interestingly, both Drp1-traps showed the enrichment of cytoplasmic or mitochondrial interactors compared to the NC (**Figure 11D**). In addition, CDGSH iron-sulfur domain-containing protein 1 (CISD1) and Histone deacetylase 6 (HDAC6), which were previously reported to interact with Drp1^{351,352}, were significantly enrichment by both Drp1-traps (**Appendix I: Tables S2 and S3**³³⁶). However, class A enrichment of peroxisomal proteins such as hydroxysteroid dehydrogenase-like protein 2 (HSDL2) or peroxisomal multifunctional enzyme type 2 (HSD17B4) was only observed for the D63-trap (**Figure 11D and Appendix I: Tables S2 and S3**³³⁶).

Notably, incubating HeLa or U2OS cell lysates with the Drp1-traps and subsequent proteomics of the bound fractions yielded comparable results (**Appendix I: Figures S5 and S6**³³⁶). Interestingly, we observed an additional enrichment of Drp1 isoform 2 in HeLa cells and Drp1 isoform 4 or 8 in U2OS cells for both Drp1-traps (**Appendix I: Figure S5F,G and S6F,G**³³⁶). These results are likely due to different expression patterns of Drp1 isoforms in the tested cell lines. Furthermore, the datasets showed enrichment of slightly different potential Drp1 interaction partners (**Appendix I: Tables S4 and S5**³³⁶).

In summary, these findings demonstrated that the Drp1-traps significantly enriched endogenous Drp1 from different cell lines together with potential interaction partners.

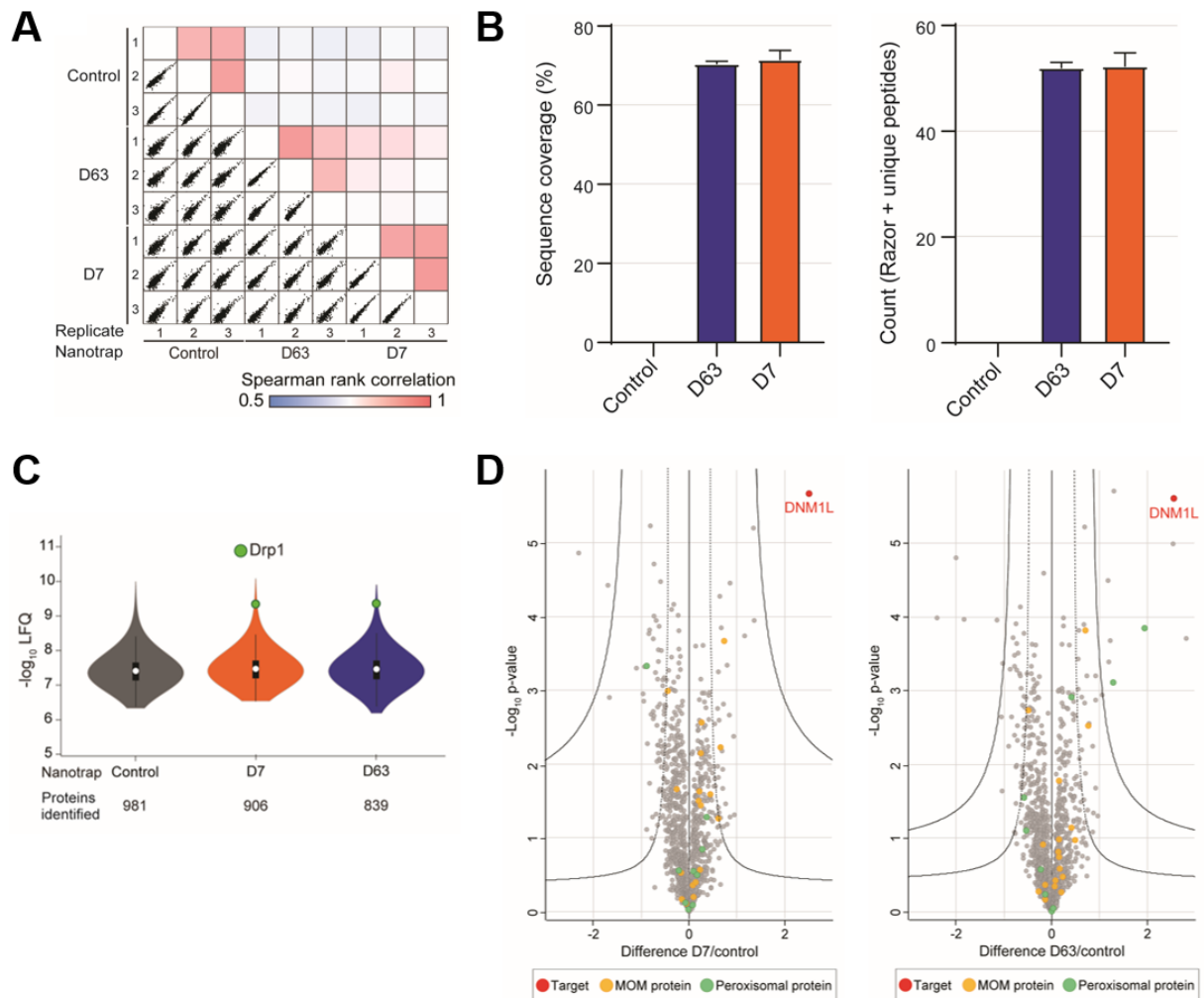


Figure 11: Analysis of the D7- and D63-trap interactomes by MS

MS analysis of immunoprecipitation using Drp1-traps (D7 and D63-trap) or GFP-trap (non-specific trap, NC) from HEK293 cell lysates. **A** Multi Spearman correlation between replicates and traps **B** Averaged sequence coverage of Drp1 after precipitation (left) and averaged identification of Drp1 razor and unique peptides corresponding to isoforms 1, 5, 6 or 7 (right) with indicated traps from three technical replicates ($n=3$) $\pm$ SD **C** Violin plot of the averaged label-free quantification intensities of the proteins identified from immunoprecipitation using HEK293 cell lysate. White circles indicate the median; box limits show the 25th and 75th percentiles as determined by R software; whiskers extend 1.5x the interquartile range from the 25th and 75th percentiles; polygons represent density estimates of data and extend to extreme values. Drp1 is labelled in green. **D** Multi-volcano analysis (Hawaiian plot) of the pulldown of D7- (left) and D63-trap (right) against the NC. $\log_2$ transformed ratios of the difference between the Drp1- and the control traps (x axis) are plotted against the $\log_{10}$ transformed P-value (y axis). Drp1 is marked in red, MOM proteins are labelled in yellow and peroxisomal proteins in

Figure 11 (continued): green. Significant interactors can be class A hits (higher confidence, $s_0 = 0.1$, $FDR = 0.01$) or class B hits (lower confidence, $s_0 = 0.1$, $FDR = 0.05$), thresholds are displayed as a solid line and a dashed line, respectively. Data analysis and figure preparation by C. Cavarischia-Rega, adapted from³³⁶.

Bivalent D7 (bivD7) enables immunofluorescence imaging of endogenous Drp1

Besides functionalising the Drp1-Nbs into affinity-capture tools for proteomic analysis of the Drp1 interactome, another goal was to establish them as immunofluorescent imaging probes. However, as noted above, neither of the two Drp1-Nbs was initially able to stain endogenous Drp1 in fixed cells³³⁵. Based on the observation that both Nbs can bind endogenous Drp1 as traps, the Drp1-Nbs were optimised for their use in immunofluorescence (IF).

First, D7 and D63 were retested as primary binding molecules in combination with a fluorescently labelled anti-VHH Ab in fixed and permeabilized GFP-Drp1 overexpressing U2OS cells. Here, D7 but not D63 showed colocalization with GFP-Drp1 (**Appendix I: Figure S7**³³⁶). When tested for detection of endogenous Drp1 in U2OS cells, no Drp1-specific signals were observed for either D7 or D63 (**Figure 12A**). Previously, it was shown that a bivalent Nb can outperform its monovalent counterpart due to increased avidity^{285,337,353}. Thus, bivalent formats of D7 (bivD7) and D63 (bivD63) were generated by genetically fusing two D7 or two D63 Nbs head-to-tail connected by a flexible Gly-Ser linker [(G₄S)₄]. For site-directed labelling, a sortase-tag was C-terminally inserted. Both bivalent Nbs were produced as secreted proteins from ExpiCHO cells and subsequently purified by IMAC (**Figure 12B**). Next, their binding affinities were determined by BLI. For bivD7, the affinity increased to a subnanomolar K_D due to reduced dissociation, whereas the affinity of bivD63 did not improve compared to D63 (**Figure 12C, Appendix I: Figure 1C and Figure S2**³³⁶).

When tested for their suitability for IF, the bivalent formats indeed outperformed D7 and D63. In GFP-Drp1 overexpressing U2OS cells, both bivalent Nbs colocalised with GFP-Drp1 and bivD7 showed a profoundly higher signal intensity compared to D7 (**Appendix I: Figure S9**³³⁶). Next, we tested bivD7 and bivD63 for staining of endogenous Drp1 in U2OS cells. Here, only the bivD7 showed a clear colocalization with the anti-Drp1 Ab (**Figure 12D**). To further confirm the recognition of Drp1 by bivD7, we performed IF imaging of wild type (wt) and Drp1 knock-out (KO) HeLa cells. While

bivD7 colocalized with the anti-Drp1 Ab in wt cells, no Nb-derived signal was detected in Drp1 KO cells (**Figure 12E**). Therefore, we concluded that bivD7 is suitable to visualize endogenous Drp1 in IF applications.

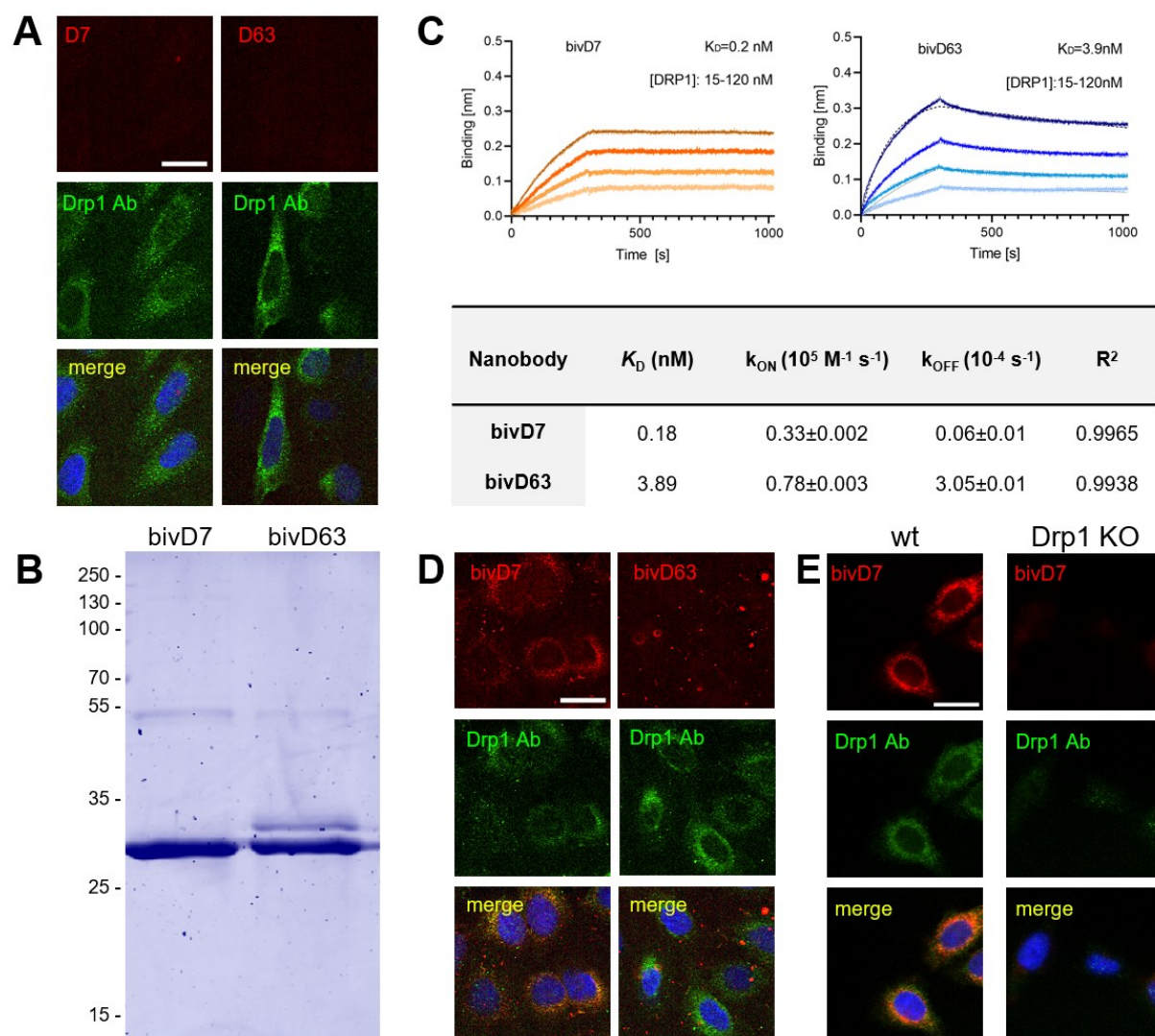


Figure 12: Bivalent Drp1-Nbs for fluorescence microscopy

A Representative confocal laser scanning microscopy images of U2OS cells stained with D7 and D63 Nbs as primary labelling probes detected with an Cy5-labelled anti-VHH antibody. As positive control, cells were co-stained with anti-Drp1 antibody (Drp1 Ab) followed by detection with an AlexaFluor488-labelled secondary Ab. **B** Recombinant expression and purification of bivD7 and bivD63 from ExpiCHO cells by immobilized metal affinity chromatography (IMAC). Coomassie staining of purified bivalent Nbs (2 μ g) is shown with molecular weight indicated on the left (in kDa). **C** Bi-layer interferometry (BLI)-based affinity measurements for bivD7 (left)

Figure 12 (continued): and *bivD63* (right). Biotinylated Nbs were immobilized on streptavidin sensors. Kinetic measurements were performed using four concentrations of purified Drp1 ranging from 15-120 nM (displayed with gradually darker shades of colour). The binding affinity (K_D) was calculated from global 1:1 fits indicated as dashed lines. The table summarizes the affinity analysis of *bivD7* and *bivD63*. Affinities (K_D), association constants (k_{on}) and dissociation constants (k_{off}) are shown as mean $\pm$ SD. **D** Immunofluorescence (IF) detection of Drp1 in fixed and permeabilized U2OS cells after staining with *bivD7* or *bivD63* as described in A. **E** IF detection of Drp1 in fixed and permeabilized wt HeLa (left column) and Drp1 KO HeLa cells (right column) stained with *bivD7* as described in D. **A, D, E** Nuclei were counterstained with DAPI. Scale bar 25 μ m. Figure adapted from ³³⁶.

Application of bivD7 in super-resolution microscopy (SRM)

As Nbs are ten times smaller than antibodies, they have a smaller linkage error and can more easily access dense cell compartments and structures. Thus, they have been previously identified as attractive probes for SRM^{118,241,277,279,281,285}.

Therefore, we tested the performance of bivD7 in STORM. To avoid unspecific labelling of lysine residues by NHS-coupling which could negatively impact the Nb's binding properties, we pursued a site-directed labelling approach. For this, we introduced a C-terminal azide group by chemoenzymatic sortagging^{285,354}, followed by click-chemistry mediated addition of a DBCO derivative. This allows us to flexibly and specifically conjugate different dyes such as the STORM-compatible AlexaFluor647 (AF647). Subsequently, we confirmed the functionality of our AF647-labelled bivD7 (bivD7_{AF647}) by successful staining of Drp1 in wt but not Drp1 KO HeLa cells (**Appendix I: Figure S11**³³⁶).

Next, we applied the bivD7_{AF647} for super resolution imaging of Drp1. These microscopy experiments were performed and analyzed by A. Jenner. First, Drp1 was stained with bivD7_{AF647} in a cell line expressing monomeric enhanced GFP-tagged Drp1 (mEGFP-Drp1) at endogenous expression levels. Confocal imaging and subsequent colocalization analysis showed a reasonable overlap of the bivD7_{AF647} signal with mEGFP-Drp1 (Pearson's correlation coefficient of 0.73 ± 0.07) (**Figure 13A-C**). Thus, we continued with STORM imaging of mEGFP-Drp1 expressing U2OS cells stained with bivD7_{AF647} (**Figure 13D**). As expected, the reconstituted bivD7_{AF647} signal overlaid with the epifluorescence signal of mEGFP-Drp1, which was measured in the same location prior to STORM imaging. These results demonstrate the suitability of bivD7_{AF647} as an imaging probe showing sufficient brightness and specificity to label Drp1 at endogenous expression levels. Finally, endogenous Drp1 in U2OS cells was stained with bivD7_{AF647} and imaged by STORM. Hereby, localizations of both diffuse cytosolic Drp1 as well as mitochondrial Drp1 complexes were detected (**Figure 13E-G**). Thus, bivD7_{AF647} enabled the visualization of macromolecular assemblies of Drp1 at mitochondria (**Figure 13G**). In summary, this demonstrated the suitability of bivD7_{AF647} as a Drp1-specific probe with minimal linkage error for SRM imaging.

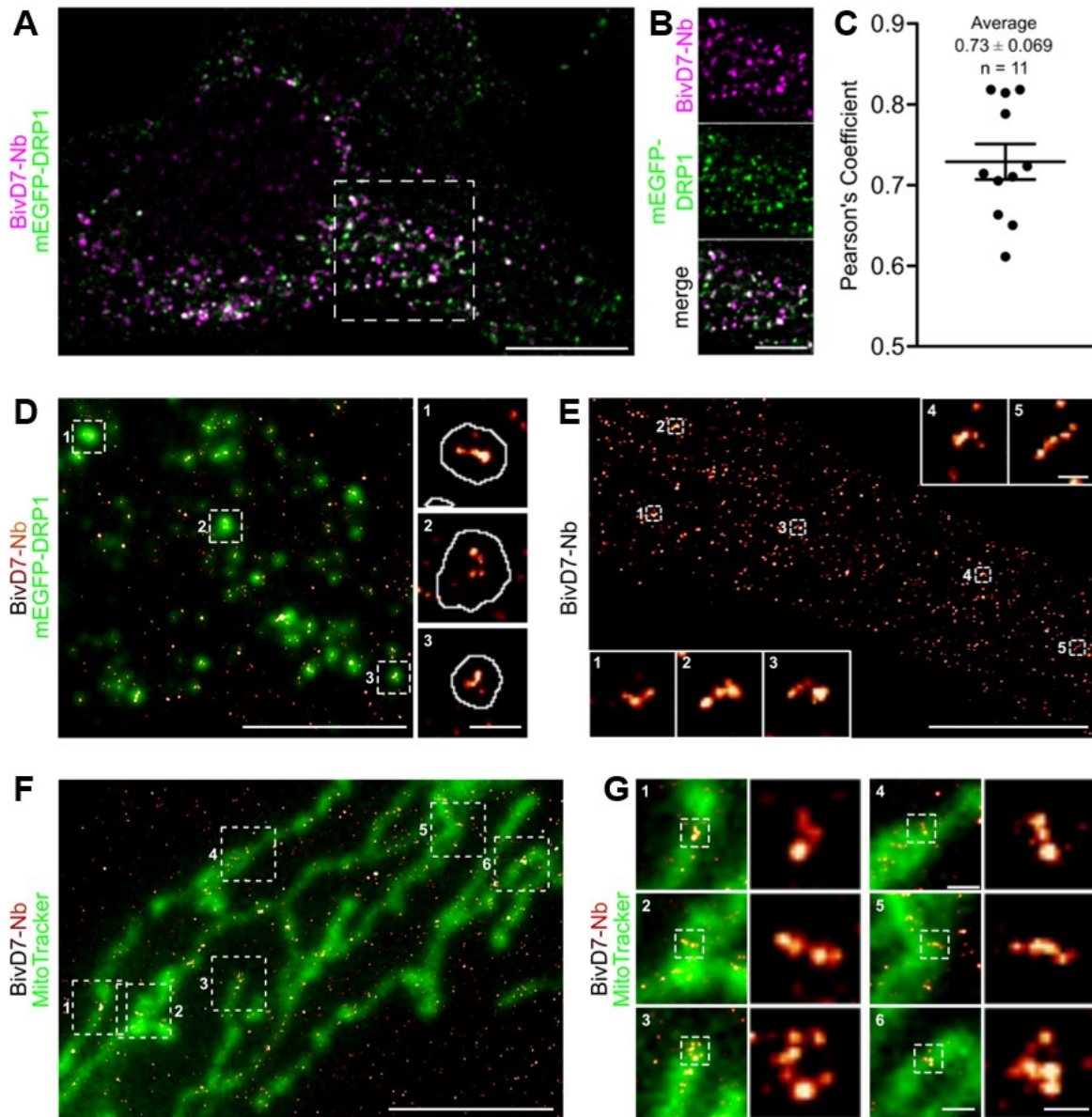


Figure 13: *bivD7_{AF647}* for super resolution microscopy

A Representative confocal fluorescence microscopy image of mEGFP-Drp1 expressing U2OS cells (mEGFP-Drp1 signal shown in green) stained with *bivD7* directly conjugated to AlexaFluor647 (*bivD7_{AF647}*; magenta). **B** Zoomed images corresponding to crop regions as indicated in A. Scale bar 10 μm , for crop 5 μm . **C** Pearson's correlation coefficient of mEGFP and *bivD7_{AF647}* fluorescence emission signals calculated from background-corrected images shown in A. Data are representative for $n = 3$ independent experiments with $n = 11$ cells. **D** Reconstructed super-resolution image of Drp1 stained with *bivD7_{AF647}* (orange-hot) overlaid with the epifluorescence signal of mEGFP-Drp1 (green). Zoomed images (right) correspond to cropped regions as indicated. White lines in the zoomed regions indicate the outline of the thresholded mEGFP-Drp1 signal. Images are representative for $n = 3$ independent experiments. Scale bar 5 μm , for zoom 500 nm. **E** Reconstructed super-resolution

Figure 13 (continued): image of Drp1 labelled with $bivD7_{AF647}$ in wt U2OS cells. Insets correspond to indicated zoomed regions. Scale bar 5 μm , for insets 200 nm. **F** Reconstructed super-resolution image of Drp1 labelled with $bivD7_{AF647}$ (orange-hot) overlayed with an epifluorescence microscopy image of mitochondria stained with MitoTracker (green) in wt U2OS cells. Scale bar 5 μm . **G** Zoomed areas of the image shown in **F** (left) with further zoom on Drp1 assemblies (right) as indicated. Scale bar 500 nm, for zoomed images 200 nm. **D, E, F, G** Data are representative of at least $n = 3$ independent experiments. Figure was prepared by A. Jenner and taken from ³³⁶.

Turnover-accelerated Drp1-Cbs can visualize the dynamics of endogenous Drp1 in live cells

In addition to visualizing Drp1 in fixed cells, we aimed to establish intracellular Drp1-Cbs to follow the dynamics of endogenous Drp1 in live cells (**Figure 14A**). However, as mentioned above, while both Drp1-Cbs can bind to endogenous Drp1 as shown by IC-IP (**Figure 14B**), they did not colocalise with GFP-Drp1 or endogenous Drp1 (observation made by F. Fagbadebo, ³³⁵).

Hereby, it has to be considered that the Drp1-Cbs were expressed under a strong promoter resulting in a substantial excess of Cbs relative to Drp1. Consequently, the fluorescence signals of the antigen-bound Cbs can be masked by signals of unbound Cbs. This is particularly problematic for detection of Drp1, as both Drp1 and unbound Cbs are predominantly cytosolic, thus resulting in high background noise. Therefore, to improve the signal-to-noise ratio, we modified our Drp1-Cbs into turnover-accelerated versions (ta-Cbs), which are degraded more rapidly by the proteasome when unbound²⁹⁵. Then, we compared the ta-Cbs with the (unmodified) Cbs in wt HeLa and Drp1 KO HeLa cells. Quantitative imaging of living cells, co-transfected with nuclear-localized GFP (GFP-NLS) as transfection control and marker for cell segmentation, revealed significantly lower average cellular intensities for the ta-Cbs compared to the Cbs (**Figure 14C, D**). In addition, due to the lack of antigen, the signal of ta-Cbs was further reduced in Drp1 KO HeLa cells (**Figure 14C, D**). Therefore, we concluded that the modification significantly reduces the amount of unbound Drp1-Cbs leading to an improved the signal-to-noise ratio.

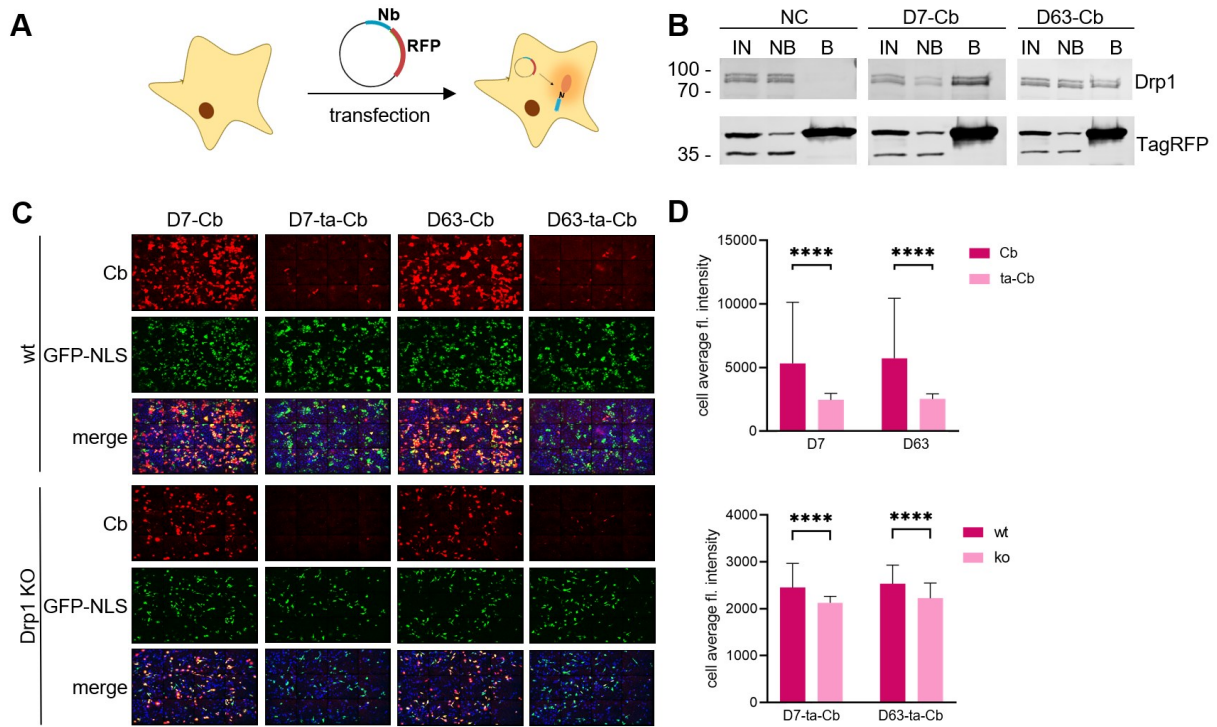


Figure 14: Generation of Drp1-chromobodies

A A plasmid encoding a chromobody (Cb) consisting of a Nb and TagRFP is transfected into cells where the Cb is expressed **B** For intracellular immunoprecipitation of Drp1, soluble protein fractions of HEK293 cells expressing the indicated chromobodies (D7-Cb, D63-Cb or an unrelated Cb as NC) were subjected to immunoprecipitation by TagRFP-trap. Input (IN), non-bound (NB) and bound fractions (B) were analysed by immunoblotting using an anti-Drp1 Ab (upper panel) and an anti-TagRFP Ab (lower panel). Molecular weights in kDa on the left. **C** Representative fluorescence images of living wt HeLa or Drp1 KO HeLa cells transiently expressing either the Drp1-Cb or the Drp1-ta-Cb constructs. A GFP-NLS encoding construct was co-transfected as transfection control. Nuclei were stained with Hoechst33242. **D** Bar chart representing the cell average fluorescence intensity of Cb signal (red channel) quantified from C. For the comparison of Cbs and ta-Cbs, significance was calculated using Kruskal Wallis test with Dunn's multiple comparisons test (upper panel). For comparing the ta-Cb intensities in wt HeLa cells and Drp1 KO cells, the significance was calculated using two-way ANOVA with Sidak's multiple comparisons (lower panel). Data are depicted as mean $\pm$ SD; n. s. $P > 0.05$, **** $P < 0.0001$; $n > 130$ cells each. Figure adapted from³³⁶.

Finally, we analysed the ability of our Drp1-Cbs and Drp1-ta-Cbs to visualize the dynamic relocation of cytosolic Drp1 to mitochondria after compound-mediated induction of mitochondrial fission. For this, we treated U2OS cells transiently expressing Drp1-Cbs or Drp1-ta-Cbs with CCCP, which uncouples mitochondrial oxidative phosphorylation resulting in Drp1 translocation to mitochondria and subsequent mitochondrial fission³⁵⁵⁻³⁵⁸. Time-lapse imaging of Drp1-Cb expressing cells showed diffuse Cb signals that did not change with CCCP treatment. In contrast, in cells expressing the Drp1-ta-Cbs an increasing number of spots became visible (**Figure 15**), which was not the case in the absence of CCCP (**Appendix I: Figure S12**³³⁶). As these spots colocalized with the MitoTracker signal, this strongly suggests that our ta-Cbs detect an accumulation of Drp1 at mitochondrial fission sites. Notably, we did not observe such patterns in Drp1 KO HeLa cells (**Appendix I: Figure S13**³³⁶), which further underscores that the detected spots are most likely Drp1 complexes bound by the Drp1-ta-Cbs. In summary, this shows that our Drp1-ta-Cbs are a suitable tool to study the accumulation of endogenous Drp1 at the MOM during fission in living cells.

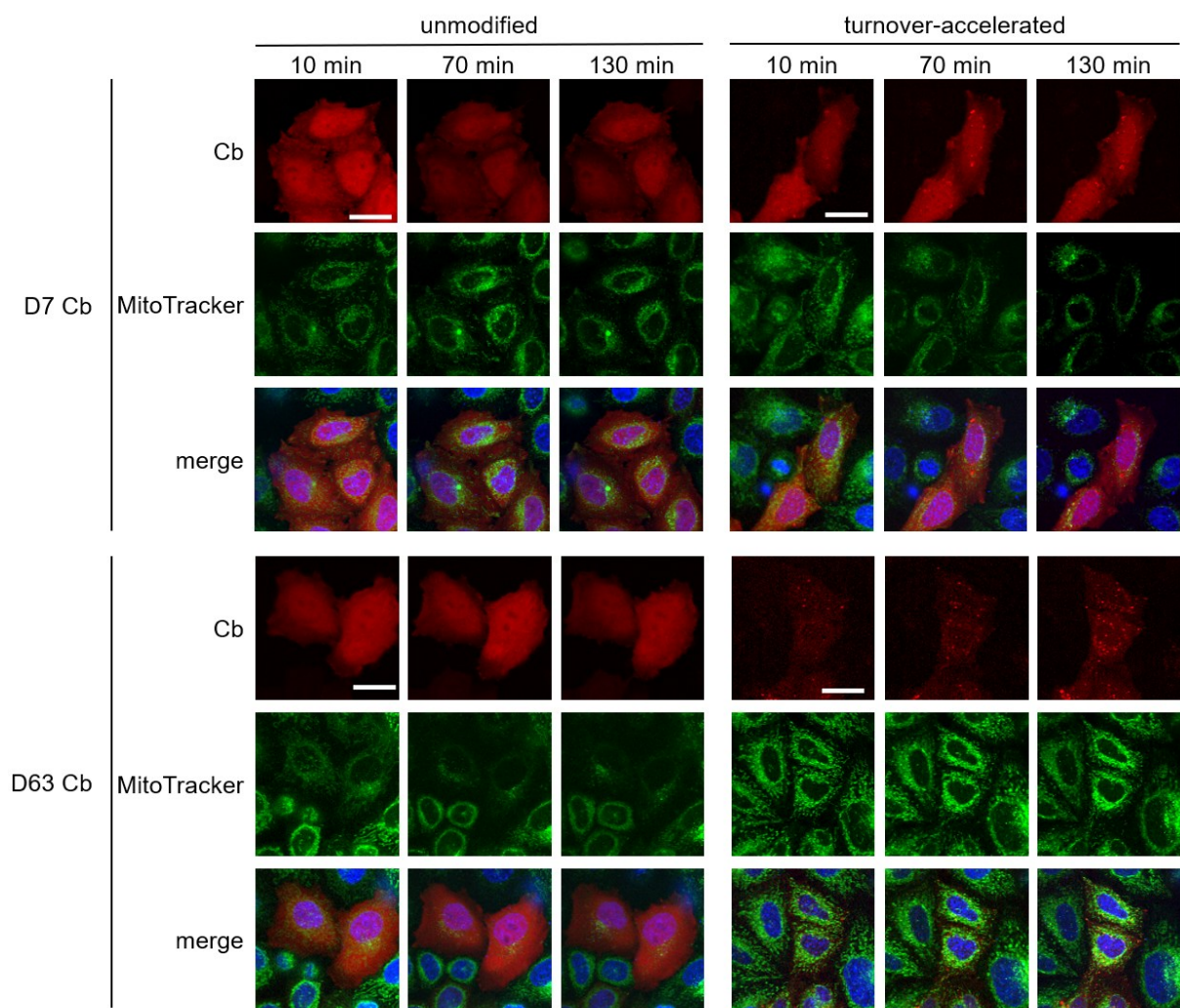


Figure 15: Time-lapse microscopy using Drp1-chromobodies

Fluorescent time-lapse microscopy of wt HeLa cells transiently expressing unmodified or turnover-accelerated chromobodies (Cbs) (red). 24 h after transfection, cells were stained with MitoTracker green (green) and Hoechst33242 (blue) and subsequently treated with 20 μ M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Representative confocal images were taken after 10, 70, and 130 min of treatment. Brightness and contrast were individually set for each Cb and kept for all time points. Scale bar 25 μ m. Figure taken from³³⁶.

Generation of Dox-inducible vector and Nb-based degron design

After demonstrating that Drp1-ta-Cbs can bind and visualize endogenous Drp1 in live cells, we asked whether the Drp1-Nbs can also be engineered for targeted degradation of Drp1. Therefore, we tested two different degron approaches.

Conventional, transient transfection of cells with a plasmid encoding for a constitutively expressed degron leads to a mixture of transfected and untransfected cells as well as an asynchronous initiation of expression which complicates downstream analysis. By using an inducible expression system, degron expression can be induced synchronously at the desired starting point. This simplifies the investigation of POI depletion effects, especially when these are short-term or less pronounced.

Therefore, a Tet-on system was selected for Nb-based Drp1-degron expression, which can be activated by Doxycycline (Dox) addition to the medium. First, an existing vector was modified to contain the desired properties: (i) encoding of the entire system on one plasmid, (ii) low leaky expression and (iii) possibility of generating a stable cell line. For this, the pB-rtTA vector³³⁸ containing the reverse tetracycline transactivator (rtTA) under a UbC promoter followed by an IRES and a Neo^R/Kan^R cassette was used. This directly links the antibiotic resistance, which can be used for stable cell line generation, with the expression of the transactivator. In addition, the pB-rtTA is a piggyBac cargo vector, further facilitating genomic insertion via the piggyBac transposase. Using this vector as backbone, all elements for an inducible expression with rtTA were inserted. As promoter, a tight TRE promoter consisting of multiple tet operators was chosen³³⁹. This was followed by a new multiple cloning site (MCS) consisting of six unique restriction enzyme sites and a polyA tail-encoding sequence.

Initially, the newly generated Dox-inducible vector was tested for functionality and optimal Dox dosage. For this, GFP was cloned into the MCS, and the resulting construct was transfected into HeLa cells. 24 h post transfection, expression was induced using 0, 10, 100 or 1000 ng/ml Dox and the GFP signal was subsequently monitored for 72 h. As expected, GFP expression was dose- and time-dependent. After 4 h, first visible GFP expression was observed for 100 and 1000 ng/ml Dox-treated cells and after 8 h, 1000 ng/ml Dox-treated cells showed a strong GFP signal. Signal intensity continued to increase over time with cells exposed to lower doses of Dox expressing less GFP (**Figure 16**). Thus, this system allows for fine-tuning of expression when necessary. Since a rapid and strong induction demonstrates the functionality of

the degrons best, a Dox concentration of 1000 ng/ml was chosen for subsequent experiments.

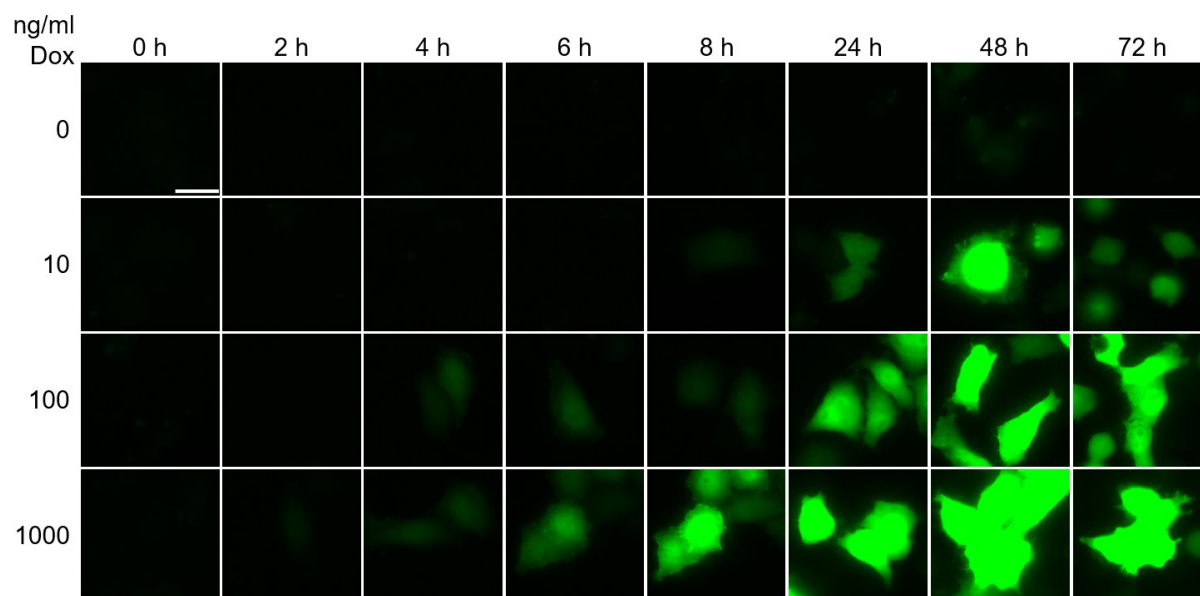


Figure 16: Determination of optimal Dox dosage for the newly generated Dox-inducible vector. GFP was inserted into the newly generated Dox-inducible vector which was then transfected into HeLa cells. 24 h post transfection, expression was induced with 0, 10, 100 or 1000 ng/ml Doxycycline and GFP signal monitored over 72 h. 25 μ m scale bar.

Following the generation and validation of the Dox-inducible expression vector, two degron systems were tested for targeted Drp1 depletion: Fbox- and RBCC-based degradation. In 2011, the combination of a GFP-binding Nb with the Fbox domain of Slmb was introduced as so-called deGradFP system for the degradation of GFP and GFP-fusion proteins³⁰⁰. Later on, we successfully adapted this approach for targeted Miro1 degradation³³⁷. However, other E3 ligases were modified for targeted degradation as well including TRIM21, which mediates proteasomal degradation of intracellular Abs and their cargo³⁰⁷⁻³⁰⁹. It has been shown that TRIM21 can be redirected to other POIs by combining its RING-, B-box-, and coiled-coil- domains (abbreviated RBCC), which are sufficient for initiating proteasomal degradation, with a Nb. This approach is known as miniTrim-Away or TRIMbody system^{309,359}.

As the intracellularly expressed D7- and D63-Cbs were able to bind endogenous Drp1, both Nbs were also tested for the generation of Drp1-specific degrons. In both F-box and RBCC-degrons, the Nb was placed N-terminally for optimal binding²²⁰ followed by the degradation domain and a C-terminal TagRFP for monitoring degron expression and localization (**Figure 17**). To ensure the steric freedom of each element, G₄S and [G₄S]₂ linkers were inserted between Nb and degron domain as well as between degron domain and TagRFP, respectively (**Figure 17**). As controls, degrons were also generated using a GFP-binding Nb (GBP)²⁸⁷ and the Pep-Nb, which binds a peptide derived from the gp120 HIV protein²⁷³.

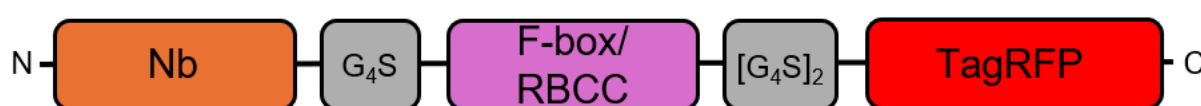


Figure 17: Schematic description of the Nb-based Drp1-degron design

The Nb-based Drp1-degrons consist of an N-terminal Nb linked by a G₄S linker to the degradation domain (F-box or RBCC) followed by a [G₄S]₂ linker and a C-terminal TagRFP.

Testing Fbox-based Drp1-degrons

For initial testing of the newly generated Fbox-based degrons, the degradation of GFP-Drp1 was monitored with GBP-Fbox-TagRFP and PepNb-Fbox-TagRFP as positive and negative control, respectively. For this, HeLa cells were co-transfected with a GFP-Drp1 encoding plasmid and the respective degron plasmid in an equimolar ratio. After 24 h, degron expression was induced and the GFP-Drp1- and the degron-signal was monitored for 0, 8, 24 and 30 h. For all constructs, degron expression was successfully induced as indicated by the TagRFP signal (**Figure 18A, B**). In GBP-Fbox-TagRFP positive cells, the otherwise diffuse cytosolic GFP-Drp1 relocated into puncta with the degron while GFP-Drp1 was depleted outside these puncta (**Figure 18B**, white arrows) indicating that GBP-Fbox-TagRFP can bind to its target. No punctate signals were observed in either the Drp1Nb- or the PepNb-Fbox-TagRFP degrons. Instead, both Nb-Fbox-TagRFP and GFP-Drp1 showed a diffuse distribution (**Figure 18**). When quantifying the GFP-Drp1 signal, however, neither the GBP- nor the Drp1Nb-Fbox-TagRFP degrons showed a reduction of signal compared to the negative control or over time (**Figure 18C**). From these findings, it has to be concluded that the Fbox-based degrons cannot degrade GFP-Drp1.

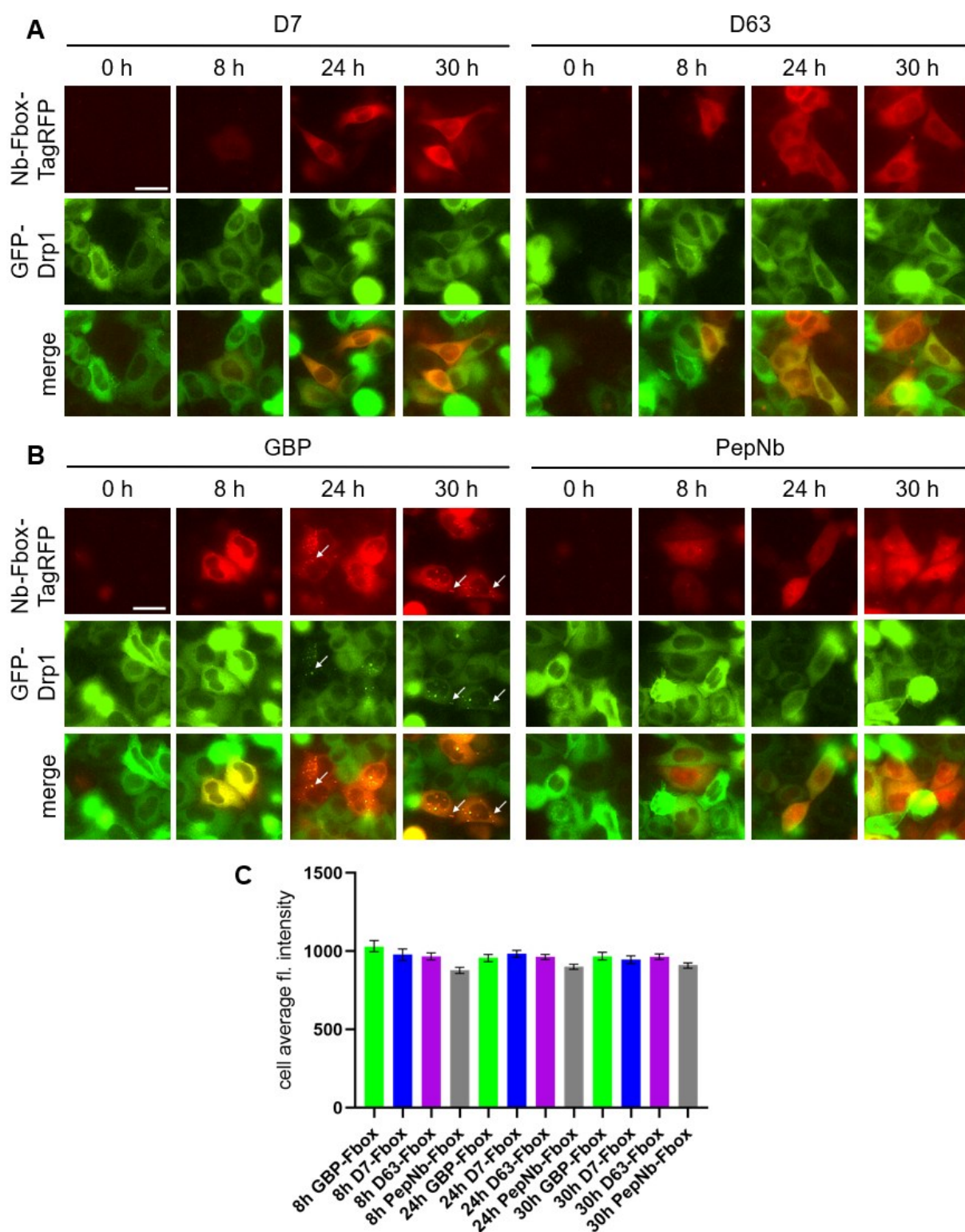


Figure 18: Functionality test of Nb-Fbox-TagRFP degrons

HeLa cells were co-transfected with Nb-Fbox-TagRFP and GFP-Drp1 encoding plasmids. 24 h post transfection, expression of degrons were induced with 1 μ g/ml Doxycycline. Images were taken 0 h, 8 h, 24 h and 30 h post induction for Drp1-degrons (A) or GFP-Fbox-TagRFP and PepNb-Fbox-TagRFP as positive and negative control, respectively (B). Exemplary puncta are

Figure 18 (continued): marked with white arrows. **C** GFP-Drp1 signal in TagRFP-positive cells was determined ($n > 1268$ cells in 4 biological replicates). Scale bar 25 μm .

Testing RBCC-based Drp1-degrons

Like the Fbox-degrons, the RBCC-degrons were tested first for degradation of overexpressed GFP-Drp1 (**Figure 19**). Here, however, the Pep-Nb-RBCC-TagRFP degon seemed to be toxic as degon-positive cells (indicated by TagRFP signal) started to round and detach (data not shown). Therefore, degradation was determined by GFP-Drp1 signal over time and compared to non-degon-expressing cells.

Like GBP-Fbox-TagRFP, GBP-RBCC-TagRFP showed co-localization in puncta with GFP-Drp1 at 8 h. With prolonged degon induction, these GBP-RBCC-TagRFP puncta disappeared and the degon resumed diffuse localization in the cytoplasm while both cytosolic and punctate GFP-Drp1 signals were clearly reduced (**Figure 19B**).

For the D7- and D63-RBCC-TagRFP, the effect was less evident. Both showed Nb-RBCC-TagRFP puncta although less pronounced than for GBP-RBCC-TagRFP (**Figure 19A, B**, white arrows). Due to the variability of GFP-Drp1 expression between cells, it was difficult to determine without quantification if degradation occurs.

For quantification, the overall GFP signal was determined for both Dox-induced and Dox-uninduced wells. This revealed that the GFP signal increased over time without degon induction (**Figure 19C**) indicating that 32 h after transfection (8 h post induction), the steady-state-level for GFP-Drp1 had not yet been reached. Therefore, a direct comparison over a longer period of time is not possible; instead, an adjustment must be made to account for this increase. First, the GFP-Drp1 levels were compared for induced and uninduced wells at the end point of 30 h Dox induction. Here, GBP-RBCC-TagRFP showed a significant reduction in GFP-Drp1 compared to the uninduced well (**Figure 19C**). Both D7- and D63-RBCC-TagRFP showed a reduction as well although not significant (**Figure 19C**). Based on the TagRFP signal, a substantial number of cells carried the GFP-Drp1 plasmid but not the degon plasmid (data not shown) which possibly obscured the reduction in GFP signal. This was probably due to the larger size of the degon plasmids compared to the GFP-Drp1 encoding plasmid (10.7 kb vs 6.9 kb), thus resulting in reduced cellular uptake. To compensate for this, the GFP signal of TagRFP-positive cells was normalized to the overall GFP-Drp1 signal in the well. As the degon-positive cells are also counted in

the total, the decrease cannot be seen as a total reduction, but rather an abstract value to determine if significant degradation occurs or not. Here, GBP, D7- and D63-RBCC-TagRFP transfected cells showed a significant reduction in GFP-Drp1 signal after 24 h compared to 8 h (**Figure 19D**). Between 24 h and 30 h, there was no additional significant degradation (**Figure 19D**). This indicates that both Drp1-degrons can degrade GFP-Drp1. Interestingly, all degron constructs showed more than 100 % normalized GFP-Drp1 signal for 8 h (**Figure 19D**). This suggests that cells with visible expression of the degron also express more GFP-Drp1 compared to the non-degron expressing cells thus making it challenging to evaluate microscopic images quantitatively. In summary, both the positive control GBP-RBCC-TagRFP and the D7- and D63-RBCC-TagRFP were capable of degrading GFP-Drp1. Therefore, these were further investigated for their ability to degrade endogenous Drp1.

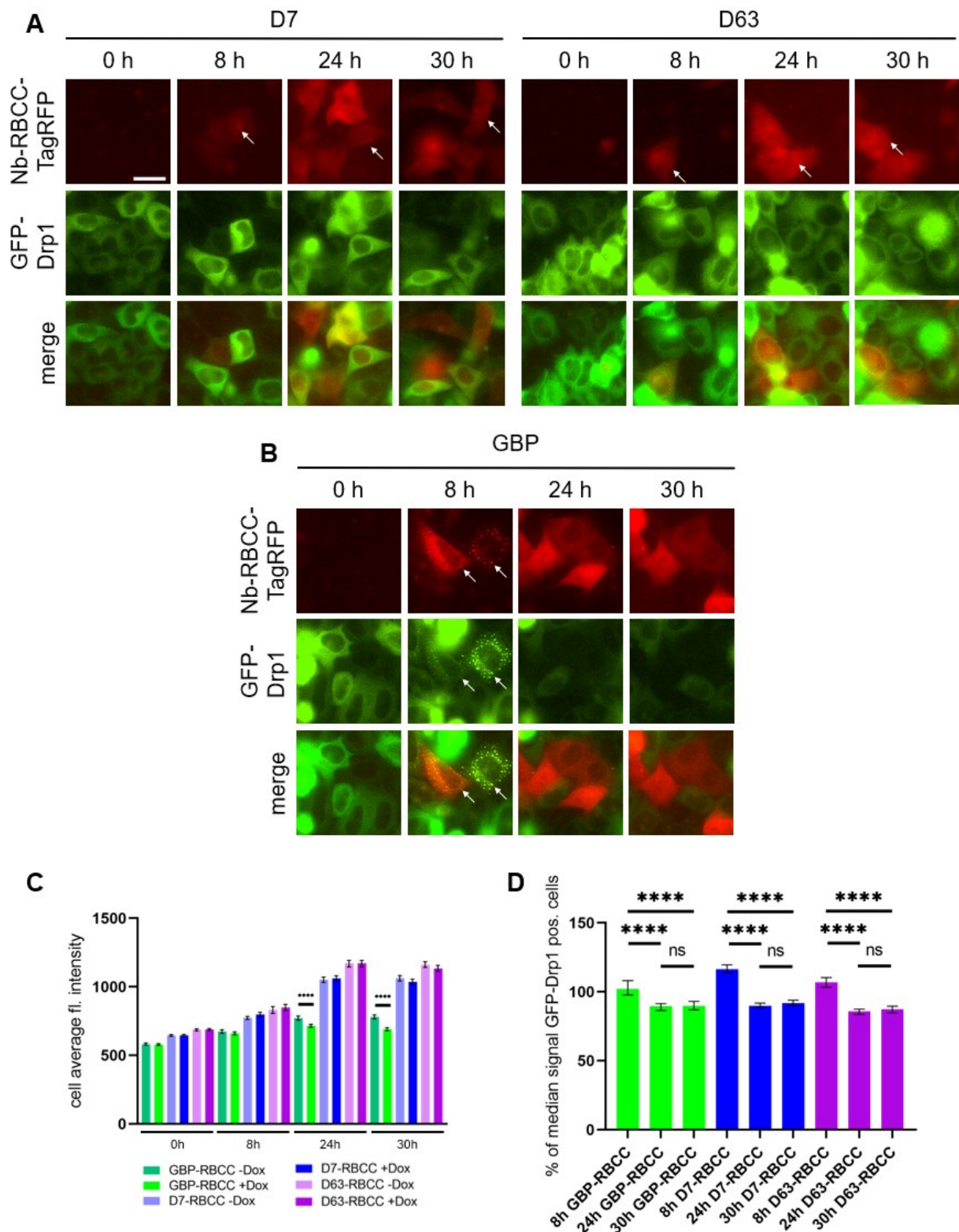


Figure 19: Functionality test of Nb-RBCC-TagRFP degrons

HeLa cells were co-transfected with Nb-RBCC-TagRFP and GFP-Drp1 encoding plasmids. 24 h post transfection, expression of degrons were induced with 1 μ g/ml Doxycycline. Images were taken 0 h, 8 h, 24 h and 30 h post induction for Drp1-degrons (**A**) or GFP-Fbox-TagRFP as positive control (**B**). **C** Average cell intensity of GFP-Drp1 positive cells in induced (+Dox)

Figure 19 (continued): and uninduced (-Dox) wells. $n > 5210$ in 3 biological replicates. Exemplary puncta are marked with white arrows. **D** GFP-Drp1 signal in TagRFP-positive cells was determined and normalized to the signal intensity of all GFP-Drp1 positive cells of the well. $n > 539$ cells in 3 biological replicates **C,D** Values depicted as mean $\pm$ 95 %CI. Significance was determined using Kruskal-Wallis-Test with Dunn's multiple comparisons test (n.s. $p > 0.05$; **** $p < 0.0001$; $n > 539$ cells in 3 biological replicates). Scale bar 25 μ m.

Degradation of endogenous Drp1

To test if the Drp1-degrons can degrade endogenous Drp1, wt HeLa cells were transfected with the degron constructs and compared to Drp1 KO cells (positive control). Starting from 24 h post transfection, cells were treated with Dox for 0, 8, 24, or 30 h. Afterwards, mitochondrial morphology was analysed by MitoTracker green (MT green) staining and Drp1 levels were determined by fixation and subsequent staining with Drp1 Ab (**Figure 20**).

As seen before with Pep-Nb-RBCC-TagRFP, GBP-RBCC-TagRFP showed cell toxicity (data not shown) hinting towards toxic side effects of the degrons in absence of the target. When analysing the microscopy images, both D7- and D63-RBCC-TagRFP showed a slight Drp1 degradation (**Figure 20A**) but no visible alteration in mitochondrial morphology (**Figure 20B**). In addition, the Drp1 Ab signal was quantified for TagRFP-positive cells. Here, D7-RBCC-TagRFP showed a significant reduction between 8h and 24h as well as further significant reduction from 24h to 30h. For D63-RBCC-TagRFP, there was a significant reduction from 8 h to 24 h as well, but not further significant reduction from 24 h to 30 h (**Figure 20C**). In conclusion, these findings indicate that both D7- and D63-RBCC-TagRFP degrons could degrade endogenous Drp1.

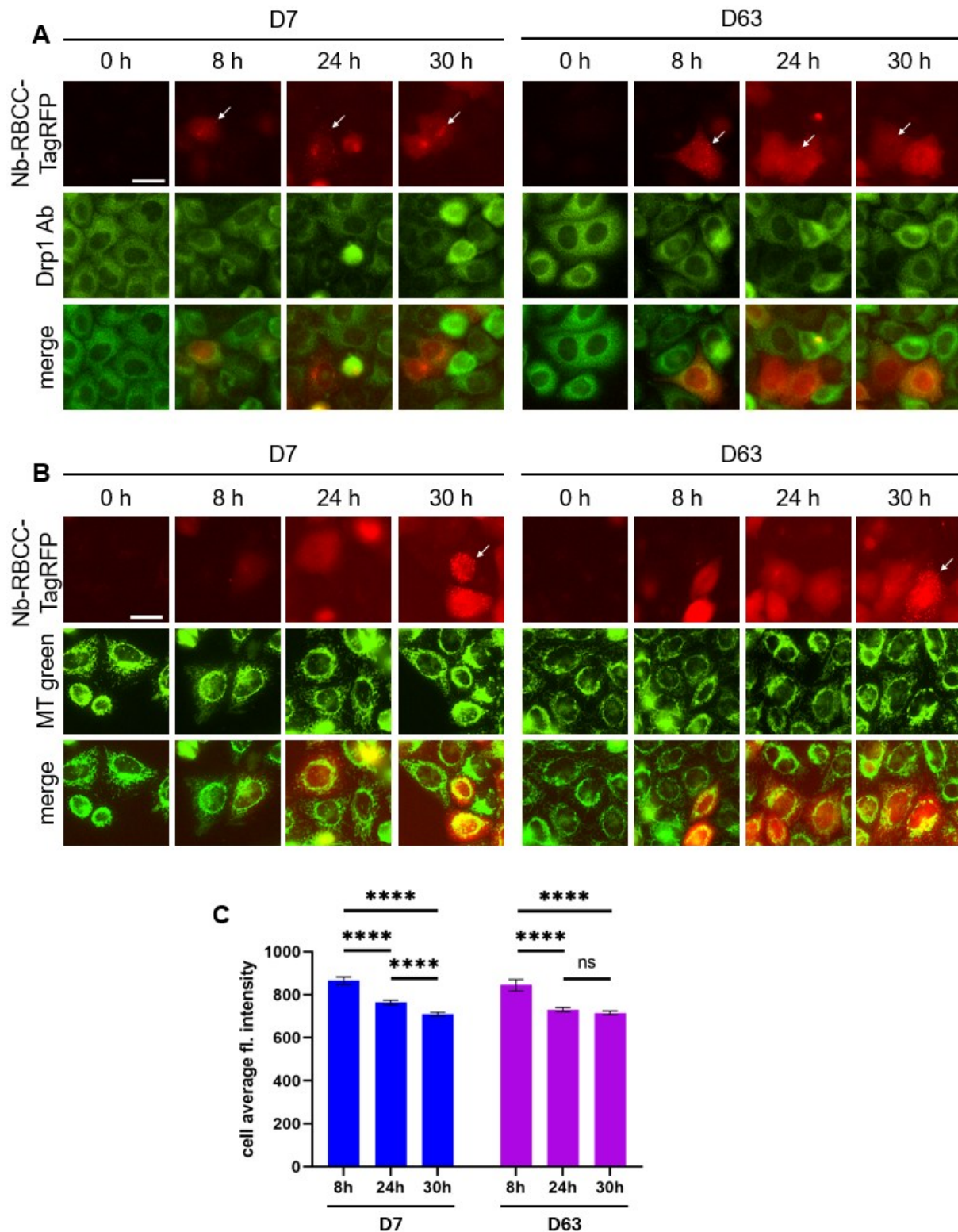


Figure 20: Functionality test of Nb-RBCC-TagRFP degrons for endogenous Drp1

HeLa cells were transfected with Nb-RBCC-TagRFP encoding plasmid. Expression of degrons were induced with 1 μ g/ml Doxycycline for 0 h, 8 h, 24 h and 30 h. Then, cells were fixed and stained with anti-Drp1 Ab (A) or stained with MitoTracker green (B). Exemplary puncta are marked with white arrows. C Average cell fluorescence intensity of TagRFP-positive anti-Drp1 Ab-stained cells. Values depicted was mean +95 %CI. Significance was determined using

Figure 20 (continued): Kruskal-Wallis-Test with Dunn's multiple comparisons test (n.s. $p > 0.05$; *** $p < 0.0001$; $n > 1735$ cells in 2 biological replicates). Scale bar 25 μm .

3.2 Results Miro1

Parts of this chapter were previously published in *Frontiers in Molecular Biosciences* under the terms of the Creative Commons Attribution License (CC BY) (published 11.3.2022)³³⁷:

A Nanobody-Based Toolset to Monitor and Modify the Mitochondrial GTPase Miro1

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Author contributions:

Author	Author position	Scientific ideas %	Data Generation %	Analysis & Interpretation %	Paper Writing %
Theresa Fröhlich	6	5	10	5	5
Title of paper:		A Nanobody-based Toolset to Monitor and Modify the Mitochondrial GTPase Miro1			
Status in publication process:		published			

F. Fagbadebo and U. Rothbauer designed the study. N. Bartlick provided recombinant Miro1. S. Nüske and A. Scholz immunized the alpaca. I (T. Fröhlich), F. Fagbadebo, P. Kaiser, T. Wagner, B. Tränkle and G. Jarjour performed all cellular and biochemical experiments. For context, the results my work is built upon are summarised in section “Generation and characterisation of Miro1-Nbs”. Specifically, I cloned the bivM114 based on a strategy developed by F. Fagbadebo and P. Kaiser. I produced the bivM114 by ExpiCHO expression and performed the subsequent purification. I functionalized the bivM114 into bivM114-trap together with F. Fagbadebo. F. Fagbadebo produced M41-, M114-, and bivM41-traps. Together with F. Fagbadebo, I performed immunoprecipitations with all four Miro1-traps. K. Zittlau and B. Maček

performed and analysed mass spectrometry experiments. F. Fagbadebo and U. Rothbauer wrote the manuscript with the help of all authors, including myself. This published manuscript was also included in F. Fagbadebo's dissertation submitted to the Faculty of Science at the University of Tübingen in 2022³³⁵.

Generation and characterization of Miro1-Nbs

Miro1 is involved in mitochondrial transport, calcium signalling and mitochondrial quality control³⁴. Failure to degrade Miro1 after mitochondrial depolarization was identified as a common feature in both familial and sporadic PD and is emerging as both prognostic marker and target for therapeutic interventions¹⁹³⁻¹⁹⁵. However, open questions regarding Miro1 function and regulation remain. To provide new tools for studying Miro1, we generated and functionalized Miro1-specific Nbs.

For this, an alpaca was immunized with recombinant Miro1. After confirmation of an immune response by serum ELISA (**Appendix II: Figure S1**^{335,337}), PBMCs were isolated from the alpaca's blood and used for the generation of a phagemid library as described above (section 3.1). From this library, seven unique Nbs (M11, M25, M41, M85, M114, M119 and M189) with diverse CDR3s were selected by phage display (**Appendix II: Figure 1A**^{335,337}). These Nbs were produced in *E.coli* followed by IMAC and SEC purification (**Appendix II: Figure 1B**^{335,337}). Binding affinity measurements by BLI showed that all selected Nbs bind with high affinities ranging from 1.9 to 29.5 nM (**Appendix II: Figure 1C, S3**^{335,337}).

To test the functionality of the Miro1-Nbs, they were covalently bound to Sepharose beads via NHS-coupling, thereby generating Miro1-traps. These traps were tested for their ability to precipitate endogenous Miro1 from HEK293 cell lysates. Here, M41, M114, M119 and M189 successfully precipitated Miro1 with M114 showing the highest efficiency (**Appendix II: Figure 2B**^{335,337}).

To determine the binding regions of the Miro1-Nbs, domain mapping was performed. For this, the Miro1-traps were tested for their ability to pulldown different Miro1 domain deletion constructs. This revealed that M11, M41, M85, M114 and M189 recognize epitopes in the C-terminal GTPase while M119 additionally requires the EF-hands for binding. The M25 epitope spans the N-terminal GTPase and the EF-hands. Based in these results, it was concluded that the selected Miro1-Nbs recognize different epitopes (**Appendix II: Figure 3**^{335,337}).

Bivalent Miro1-traps show higher enrichment of Miro1

As it was previously demonstrated that a bivalent format can improve a Nb's binding properties by increasing the avidity^{285,360-362}, two Miro1-Nbs were selected for further

optimization: M114 which showed the highest pulldown efficiency for endogenous Miro1 and M41 which had the highest affinity indicated by BLI measurements³³⁵.

To generate the bivM114-trap, bivM114 was first cloned, produced and purified as described for the bivalent Drp1-Nbs (see section 3.1). Then, analogously to the AF647-conjugation of bivD7, a C-terminal azide group was introduced by sortagging^{285,354}, followed by click-chemistry mediated addition to dibenzocyclooctyne (DBCO)-agarose beads. The bivM41-trap was produced accordingly by F. Fagbadebo.

Next, we compared the bivM41- and bivM114-traps with their monovalent counterparts (M41-trap and M114-trap, respectively) by determining the pulldown efficiencies for endogenous Miro1 from HEK293 cell lysates (together with F. Fagbadebo and K. Zittlau). In immunoblotting, bivM41-trap showed a considerably higher enrichment of Miro1 compared to M41-trap while the bivM114-trap only resulted in a slightly higher enrichment compared to the monovalent trap (**Figure 21A**). Both bivalent Miro1-traps showed higher signal intensities in their bound fractions compared to the positive control (Miro1 Ab, **Figure 21A**).

Like for the Drp1-traps, we determined which proteins besides Miro1 were enriched by MS analysis in three technical replicates. With a Spearman rank correlation close to one (**Figure 21B**), the reproducibility was high between replicates. However, the correlation was lower between NHS- and sortase-coupled traps indicating an influence of the used matrix. In addition, the bivalent traps showed a higher background of up to 1,760 proteins, which is comparable to the controls. Next, we analysed the enrichment of Miro1 (encoded by *RHOT1*). In total, 31 “razor” peptides of Miro1 isoform 3 and 30 “razor” peptides of isoform 2 including one unique peptide, were identified (**Appendix II: FigureS8C**³³⁷). Except for M41 trap, where only isoform 3 was captured, both isoforms 2 and 3 were detected (**Appendix II: FigureS8D**³³⁷). In addition, no structurally related proteins of the Ras GTPase family were enriched indicating a Miro1-specific binding (**Appendix II: Supplementary Table S3**³³⁷). Our data further revealed that more Miro1 peptides were identified and a higher sequence coverage was achieved when using the bivalent traps (**Figure 21C**).

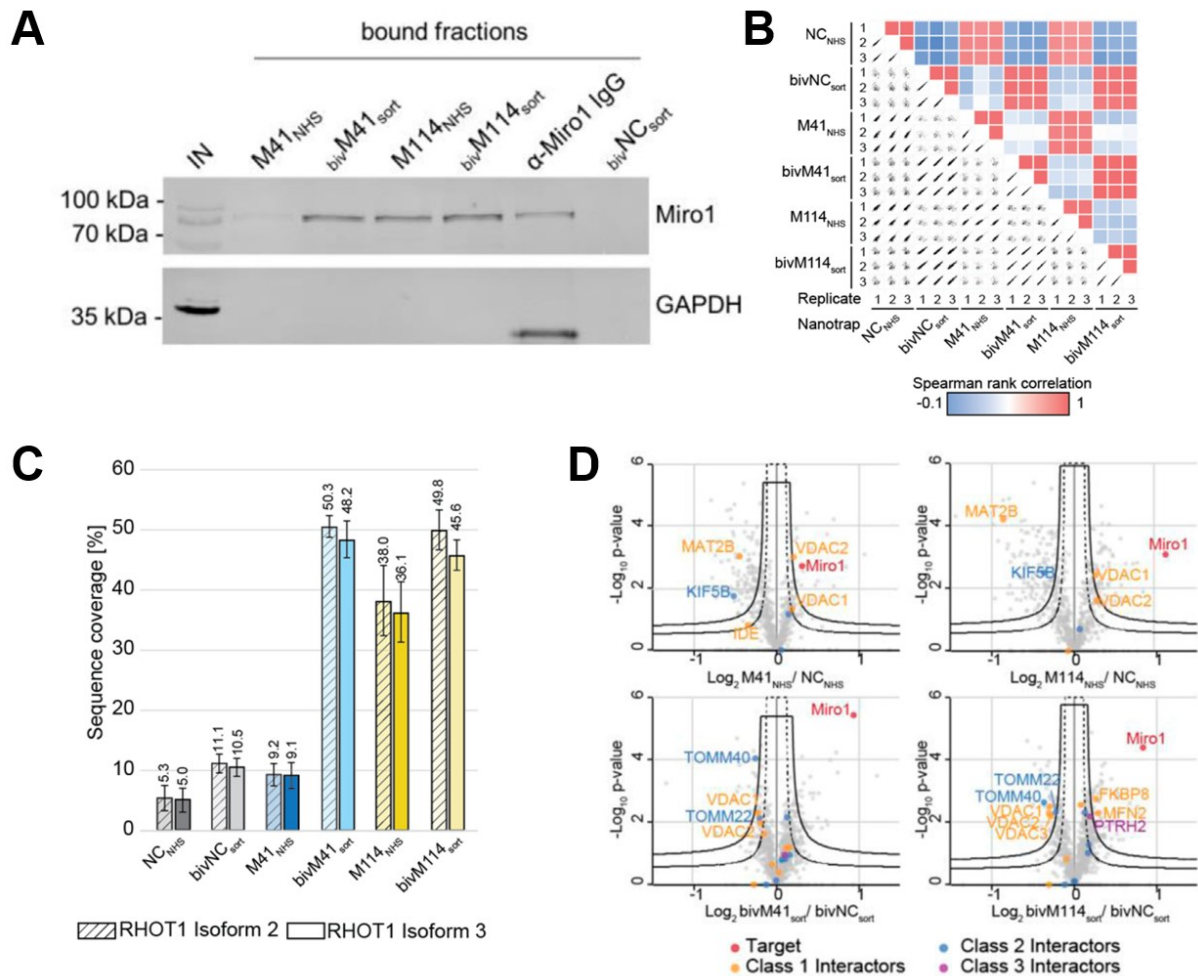


Figure 21: Analysis of the Miro-trap interactomes by MS

The soluble protein fraction of HEK293 cells was incubated with the M41-, M114-, bivM41- and bivM114-traps and the bound fractions analysed by immunoblotting and MS (in 3 technical replicates). **A** Representative immunoblot. Anti-Miro1 Ab bound to Protein A/G beads and a non-related Nb-trap were used as positive and negative control, respectively. Input (IN) and bound fractions were blotted and stained with anti-Miro1 Ab. **B** Spearman rank correlation between replicates and traps. **C** average sequence coverage of Miro1 (RHOT1) in MS analysis of bound fractions. **D** Miro1 interactor classification based on STRING database. class 1: direct interactor (confidence score >0.9), class 2: direct interactor (confidence score <0.9), class 3: indirect interactor (confidence score <0.9). Figure adapted from³³⁷ (Originals prepared by F. Fagbadebo and K. Zittlau).

Next, we analysed which known Miro1 interactors (based on STRING database annotation) were also enriched. While known first shell physical interactors of Miro1 (see **Figure 6**) were not significantly enriched, bivM114-trap significantly enriched the known interactor Mitofusin 2 (MFN2)³⁶³ (**Figure 21D, Figure 22**). In summary, the generation of bivalent formats resulted in an improved performance of the Miro1 traps for affinity capturing Miro1. Especially the bivM114-trap might be a useful capture reagent for future interactome studies of Miro1.

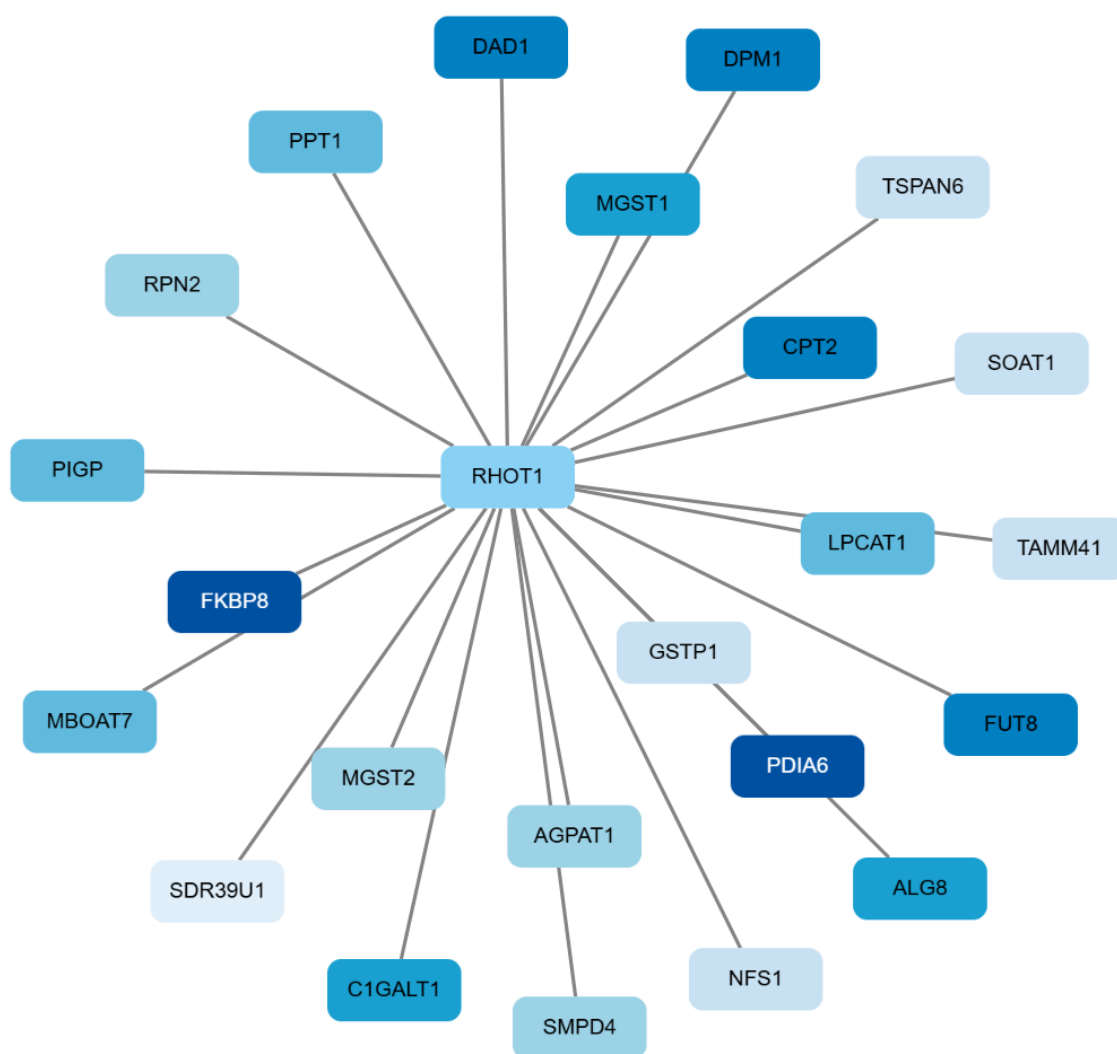


Figure 22: Significantly enriched proteins identified by bivM114-trap

Significantly enriched proteins (class A and class B) in the bound fraction of the bivM114-trap (Appendix II: Supplementary Table S3³³⁷) visualized with Cytoscape^{349,350}. Darker colour indicates higher $-\log_{10}(p\text{-value})$.

3.3 Results Testing cholesterol-coomassie for intracellular nanobody delivery

After demonstrating the successful development and functionalization of Nbs against Drp1 and Miro1, the final goal was to establish a method for the efficient transduction of these Nbs into living cells. In 2020, Tai *et al.* published a cholesterol-coomassie (cho-coo) based protein transduction method (**Figure 9**)³³⁴. As it was described as especially suited for small proteins, this was an interesting approach to test for intracellular delivery of Nbs.

To test whether fluorescently-labelled Nbs are efficiently transduced and retain their binding properties, an initial experiment using the well-described and broadly applicable bivBC2 Nb was performed. This bivalent Nb recognizes a short peptide epitope, the BC2-tag, and was previously successfully applied for STORM imaging of intracellular antigens in fixed cells²⁸⁵. Exploiting the characteristic pattern of actin fibres as simple readout, bivBC2-Nb conjugated to AlexaFluor 488 (bivBC2_{AF488}) was transduced with cho-coo into HeLa cells expressing an endogenously BC2-tagged version of actin (BC2T*ACTB*)²⁸⁵ or into wt HeLa cells. Fluorescent imaging of live cells showed the transduction of a small number of BC2T*ACTB* HeLa cells where distinct actin fibres became visible, indicating that the Nb can bind its antigen upon transduction (**Figure 23**). Based on these initial findings, I decided to further test and optimize the cho-coo transduction approach for Nb transduction.

For proof-of-principle studies, bivVB6 was chosen, a Nb that binds to endogenous vimentin²⁵³. The characteristic pattern of vimentin allows a simpler readout compared to Drp1 or Miro1. In addition, bivVB6 has been functionalized as a Cb before demonstrating the ability to bind its target in live cells²⁵³. As bivVB6 recognizes an endogenous ubiquitously expressed protein, this allowed the use of different cell lines while bivBC2-application would have been limited BC2T*ACTB* HeLa cells or would have required the generation of a new CRISPR/Cas9-modified cell line.

In the original publication introducing cho-coo, Tai *et al.* showed that the applicable cho-coo to protein ratio is dependent on the size and shape of the transduced protein³³⁴. Thus, based on the ratios for proteins with similar molecular weight determined by Tai *et al.*, the optimal Nb to cho-coo ratio was determined. 1:8, 1:12, 1:16 and 1:20 bivVB6_{AF488} to cho-coo ratios were tested in HeLa and U2OS cells. For all tested ratios, vimentin-like structures were visible (**Figure 24A**). No punctate staining, which would indicate insufficient coating of the Nb with cho-coo and

subsequent endosomal uptake, was observed (**Figure 24A**). To minimize any potential side effects by cho-coo, 1:8 was chosen as ratio for future Nb transduction experiments.

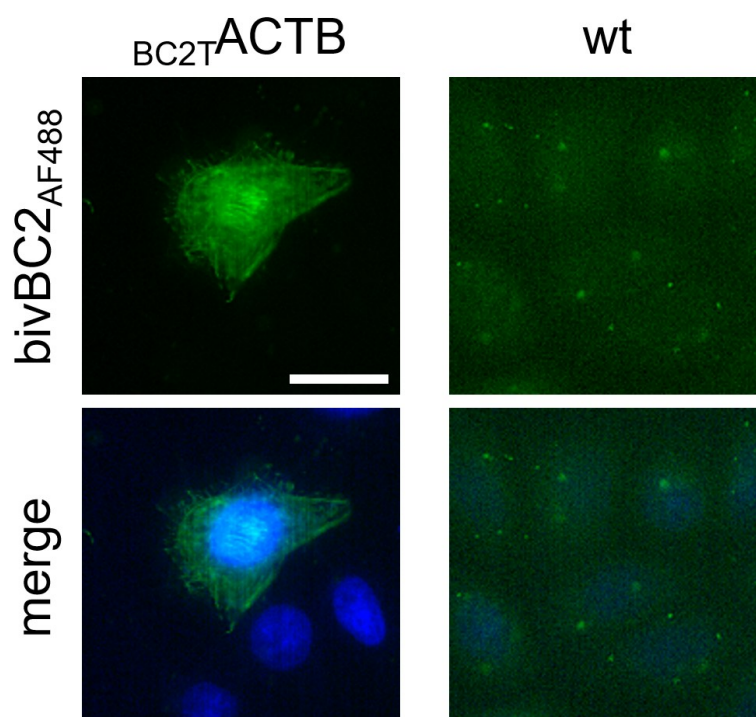


Figure 23: cho-coo mediated transduction of $BC2TACTB$ and wt HeLa cells with $bivBC2_{AF488}$

For transduction of $BC2TACTB$ HeLa cells and HeLa wt cells, these were incubated with preformed $bivBC2_{AF488}$ -cho-coo complexes (1:10 ratio) for 1 h. In parallel, nuclei were stained with Hoechst 33258. After incubation, cells were washed and imaged by fluorescence microscopy. Scale bar 25 μm .

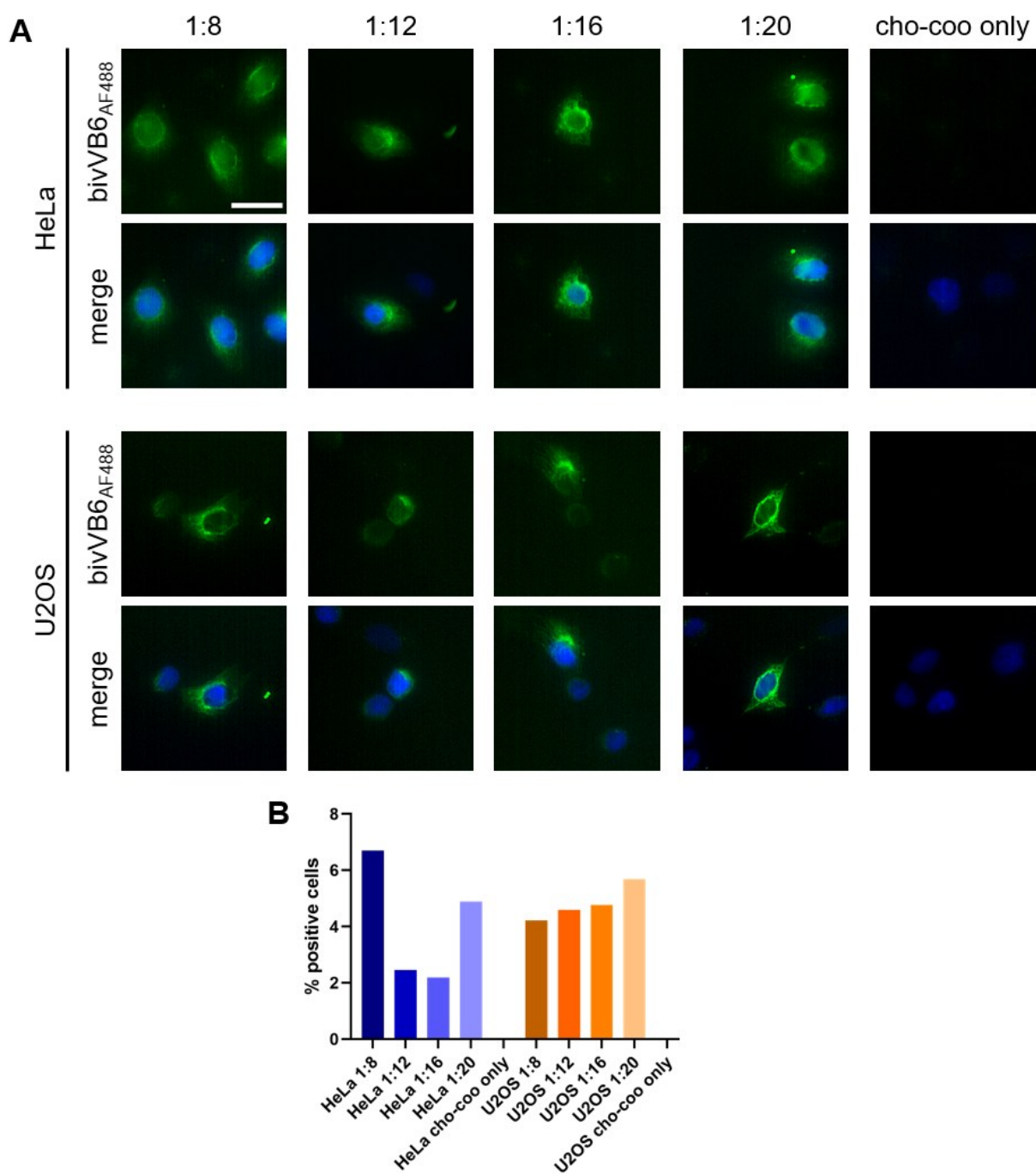


Figure 24: Optimisation of cho-coo to Nb ratio

A HeLa and U2OS cells were incubated with 100 nM bivVB6_{AF488} complexed with cho-coo in varying ratios (1:8, 1:10, 1:16 and 1:20). As negative control, cells were incubated with 2 μ M cho-coo, which is equivalent to the highest applied cho-coo concentration in this experiment. In parallel, nuclei were stained with Hoechst 33258. After 1 h incubation, cells were washed and imaged by fluorescence microscopy. Scale bar 25 μ m. **B** Percentage of cells with visible bivVB6_{AF488} staining was determined for each condition by automated image segmentation.

While a clear vimentin staining was visible in some cells, the overall transduction efficiency was low compared to a typical plasmid transfection (**Figure 24B**). In the original publication, cells were transduced at low confluency as indicated by the stated number of seeded cells per well³³⁴. Therefore, as this is unusual for fluorescence microscopy experiments, I hypothesized that the cell confluency at transduction might influence the efficiency. To test this, three different cell lines (HeLa, U2OS, and A549) were seeded with 3000, 6000 and 9000 cells/well and 24 h afterwards transduced with bivVB6_{AF488} and cho-coo. Next, the percentage of cells with a visible vimentin staining was determined. While HeLa cells showed a higher transduction efficiency with lower cells/well, this was not the case for the other two cells lines which showed no clear pattern (**Figure 25A, B**). However, no condition reached transduction efficiencies above 10 %.

Fluorescence microscopy of intracellular targets usually requires fixation and permeabilization of the cells thus making the investigation of dynamic processes challenging. As cho-coo allows the transduction of fluorescently-labelled Nbs into live cells, this method enables the monitoring of protein dynamics in the same cell over time. Thus, to explore this advantage, bivVB6_{AF488} was transduced into HeLa and A549 cells and the cellular vimentin distribution was monitored for 48 h. While non-transduced cells divided normally, the transduced cells did not divide and showed condensed nuclei as indicated by Hoechst staining (**Figure 26**). Therefore, it seems that cho-coo is either toxic to the cells or only allowed transduction into already damaged cells. Considering this, the project was not continued as a considerable bias would be introduced by investigating dying cells.

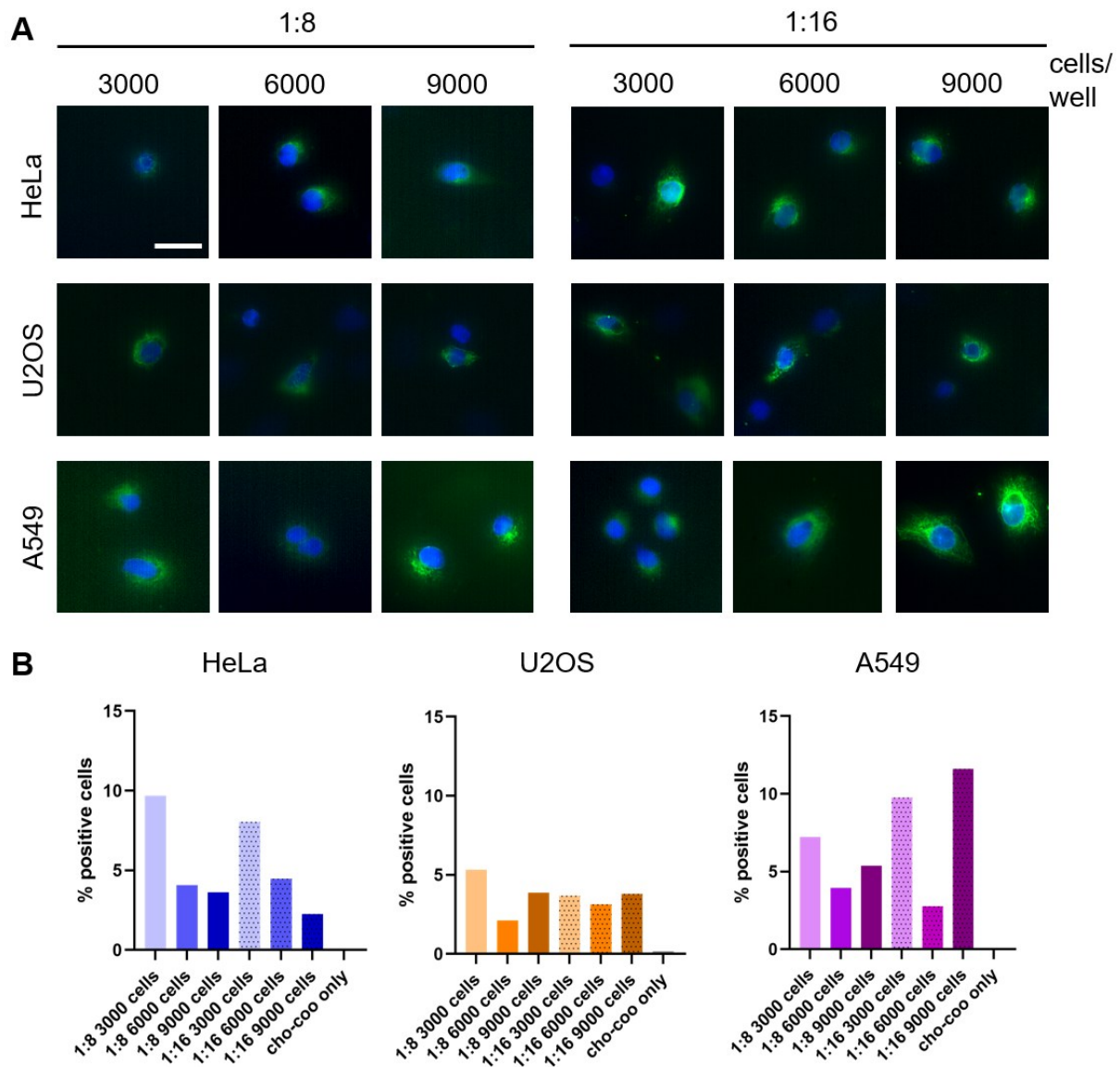


Figure 25: Determination of the influence of cell confluency on the transduction efficiency
A HeLa, U2OS and A549 cells were seeded at 3000, 6000 or 9000 cells/96-well. The next day, cells were incubated with 100 nM *bivVB6*_{AF488} complexed 1:8 or 1:16 with cho-coo for 1 h. Nuclei were stained with Hoechst333258 in parallel. Scale bar 25 μ M. **B** Percentage of cells with visible *bivVB6*_{AF488} staining was determined for each condition by automated image segmentation.

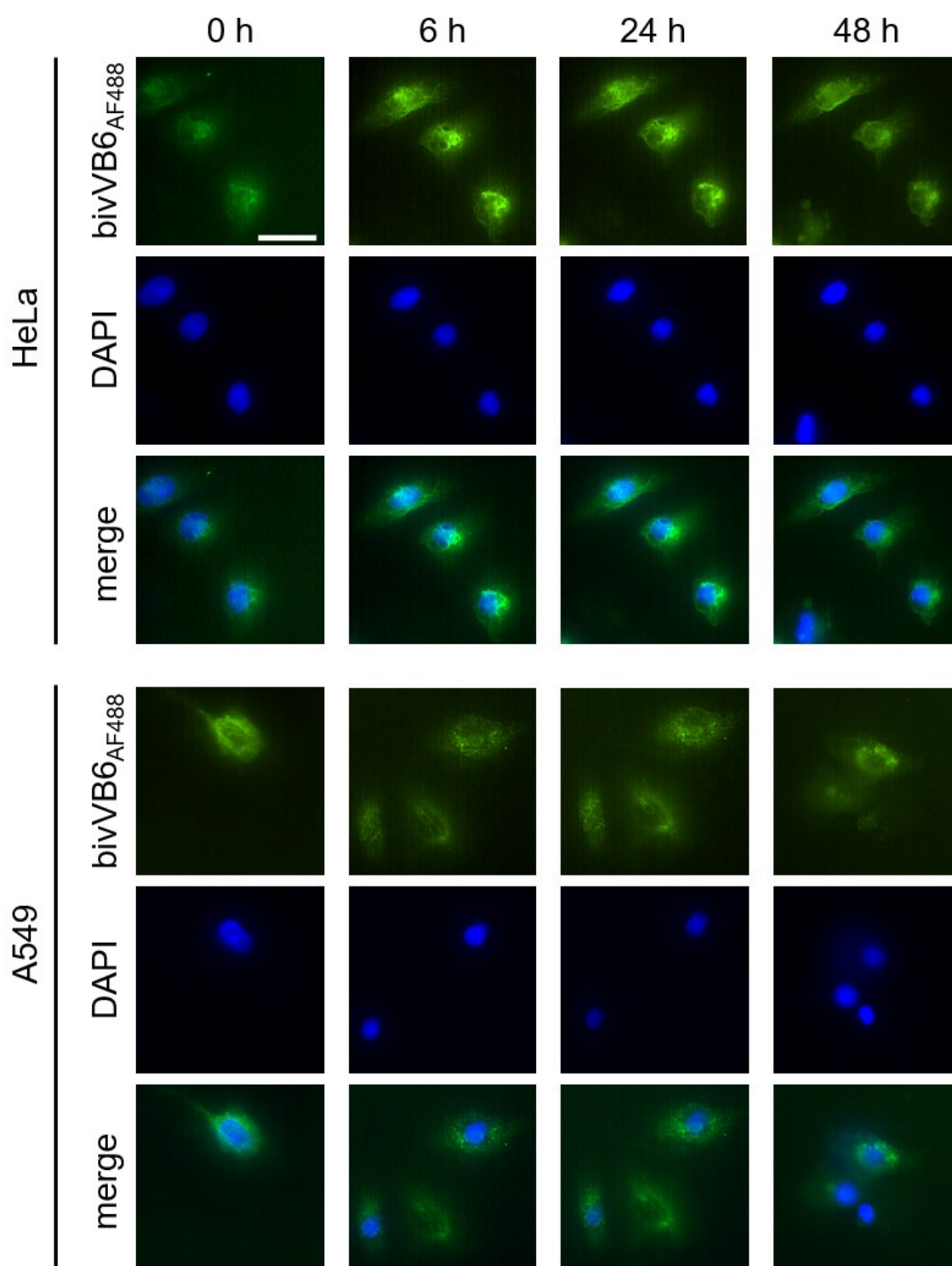


Figure 26: Time lapse microscopy of HeLa and A549 cells after cho-coo-based transduction with bivVB6_{AF488}

HeLa or A549 cells were incubated with 100 nM bivVB6_{AF488} complexed 1:8 with cho-coo for 1 h. In parallel, nuclei were stained with Hoechst333258. Cells were washed and imaged after 0 h, 6 h, 24 h and 48 h. Scale bar 25 μ m.

6 Discussion

6.1 Discussion Drp1

Drp1 is a central protein in mitochondrial fission as it executes the mitochondrial membrane constriction via its GTPase activity^{49,63}. Thus, Drp1 plays a crucial role in steady-state mitochondrial dynamics³⁶⁴ and apoptosis-related mitochondrial fragmentation¹⁰⁸. Dysregulation of Drp1 has been linked to a variety of diseases, including neurodegeneration⁵⁴, cardiovascular diseases^{156,365-367} and cancer^{91,128,129,367}. However, open questions remain regarding Drp1 mode of action and regulation^{55,60,368}.

Conventional labelling approaches using fusions of Drp1 with a FP have been shown to affect both GTPase activity and oligomerization dynamics of Drp1²¹⁶ thus limiting their suitability for answering these questions. Therefore, new advanced research tools avoiding overexpression and/or direct modification of Drp1 are needed^{216,367-370}.

To address this, I improved the Drp1-Nbs D7 and D63 by generating bivalent formats. In addition, together with my collaboration partners, I tested both monovalent and bivalent Nbs for their applicability in a variety of biochemical and cell biological research settings. By functionalisation and engineering of our Drp1-Nbs, we have successfully generated i) affinity capture matrices (Drp1-traps), ii) labelling probes for fluorescence microscopy including STORM, iii) Cbs for monitoring Drp1 in living cells and iv) Drp1 degradation tools (Drp1-degrons).

By both immunoblotting and MS-based proteomic analysis, we demonstrated that the two Drp1-traps efficiently precipitate Drp1 from whole cell lysates. Notably, the immunoblot analysis showed captured Drp1 as multiple bands being in line with previous reports^{81,87,367,371,372}. This can be attributed to the molecular weight differences between the Drp1 isoforms^{69,367,373} or PTMs^{55,99,100,374}. Therefore, applying these traps for precipitation of Drp1 from other cell types or tissues probably will result in the identification of different Drp1 isoforms or PTMs due to distinct expression patterns. Indeed, when analysing the proteome of our Drp1-traps after incubation with the soluble protein fractions of three different cell lines (HEK293, HeLa and U2OS) using MS, we found different Drp1 isoforms enriched. However, our sequence coverage was insufficient to identify the exact isoforms captured. The in-depth analysis also confirmed effective and specific enrichment of Drp1 with the identified peptides resulting in a high sequence coverage. In addition, the MS analysis revealed the enrichment of MOM- and peroxisome-associated proteins reflecting the mitochondrial

and peroxisomal localization of Drp1. Interestingly, enrichment of peroxisomal proteins was found for the D63- but not the D7-trap. This can be explained by different steric and allosteric effects as they bind to different domains of Drp1 (**Appendix I: Figure 2A**³³⁶). Overall, both Drp1-traps could precipitate endogenous Drp1 with higher efficiency than the tested commercial monoclonal anti-Drp1 Ab. Thus, the Drp1-traps could replace the commonly used immunoprecipitations using FLAG-³⁷⁵, Myc-³⁷⁶ or HA-^{98,377,378} tagged Drp1 to avoid altered Drp1 interaction due to tagging as well as increase the immunoprecipitation efficiency compared to application of the commercial Drp1 Ab³⁷⁷. Therefore, we conclude that both Drp1-traps enable a fast and efficient analysis of the Drp1-interactome.

Despite their high, nanomolar affinity for recombinant Drp1, both monovalent Nbs failed to stain endogenous Drp1 when applied for immunofluorescence labelling. This might be due to a reduced accessibility of the epitope, either in its native environment or by fixation. To improve their binding, we generated bivalent Nbs, an approach that increases the avidity^{285,360-362} and has significantly improved antigen recognition of other Nbs^{285,337,367}. Indeed, the bivalent bivD7 was able to stain endogenous Drp1 in fixed cells which was not observed in Drp1 KO cells. BivD7 was then C-terminally conjugated with the STORM-compatible fluorophore AF647 by sortagging and click chemistry. Sortagging, different to other methods like NHS-coupling, allows for highly site-specific conjugation with the resulting end product bearing a single modification at a predefined site^{379,380}. Thus, this approach is commonly used for conjugation of Nbs with various fluorophores and radiotracers^{285,379-381} and is therefore a state-of-the-art labelling approach for Nbs. We successfully applied bivD7_{AF647} for STORM imaging detecting endogenous Drp1 in the cytoplasm and on mitochondria. This site-directedly labelled bivD7_{AF647} has a small linkage error compared to the five-fold larger Abs regularly used in STORM. With technical advances further improving the resolution of SRM, the linkage error will gain importance creating a need for probes with a small linkage error like bivD7_{AF647}.

Recently, it was demonstrated that Drp1-labelling by genetic fusion with tags like FPs (e.g. GFP) alters its oligomerization state and GTPase activity consequently affecting its function^{216,367}. Thus, a tag-free approach is desirable to reduce artefacts while studying Drp1 by live-cell microscopy. In the past, Cbs were applied to visualize endogenous antigens in living cells with high spatial and temporal resolution^{253,287-290,367}. For example, the actin-Cb, different to other labelling methods like Rhodamine-

phalloidin staining, still allowed the dynamic movement of actin²⁹². Therefore, we generated D7- and D63-Cbs and applied them for monitoring the dynamics of endogenous Drp1 using fluorescence time-lapse imaging of living cells. As Drp1 is mainly diffusely localized in the cytoplasm, it was challenging to distinguish between the signals of unbound and Drp1-binding Cbs. This problem often arises when expressing genetically encoded imaging probes using a constitutive promoter^{367,382} and several approaches have been developed for reducing the levels of unbound intrabodies and Cbs^{294,295,367,383,384}. Here, aiming for faster proteasomal degradation of unbound Cbs, we generated Drp1-ta-Cbs. For the first time, these signal-to-noise ratio improved ta-Cbs enabled the live-cell imaging of the CCCP-induced relocalization of endogenous cytoplasmic Drp1 to mitochondrial fission sites.

To monitor the short-term effects of Drp1 depletion, I generated inducible Drp1 degradation tools (Drp1-degrons) using two approaches that were originally developed for the degradation of GFP; Fbox- and RBCC-based degrons^{300,309,312}.

When testing the Fbox-based Drp1-degrons along with appropriate controls, however, neither the positive control GBP-Fbox-TagRFP nor D7/D63-Fbox-TagRFP resulted in a significant reduction of GFP-Drp1 compared to the negative control PepNb-Fbox-TagRFP. As relocalization of GFP-Drp1 was induced by GBP-Fbox-TagRFP, it seems that GFP-Drp1 was recognized but not degraded. This might be due to the positioning of the Fbox domain in the degron. While other studies placed the Fbox domain N-terminally^{300,305,306,337}, analogous to its localization in its original protein Smlb³⁰⁰, I placed the Nb N-terminally for optimal target binding²²⁰. Therefore, the Fbox function might be sterically hindered.

With the RBCC-based degrons, however, I was able to degrade ~15 % for both overexpressed GFP-Drp1 and endogenous Drp1. While only a fraction of Drp1 was degraded, there are several caveats about the quantification. First, it was not accounted for the autofluorescence of cells. In addition, signals were normalized to the overall (GFP-)Drp1 signal in the well. Therefore, this quantification reflects the difference of degron-expressing cells compared to the overall (GFP-)Drp1 expressing cells rather than absolute reduction of (GFP-)Drp1. Consequently, the quantification is more suitable for qualitative than for quantitative assessment of Drp1 degradation. Additionally, an unknown percentage of Drp1 might be inaccessible for degradation by the Drp1-degrons due to steric clashes with Drp1 interaction partners. Here, binders

against exogenous proteins like GFP have an advantage that they have no natural binding partners which could block the interaction.

In recent years, different approaches have been developed for targeted degradation of whole mitochondria^{385,386} or mitochondrial matrix proteins^{387,388}. Thus, the Drp1-degrons complement the growing toolset of mitochondrial degradation tools. However, as other degrons achieved 30-60 % degradation^{337,388,389}, the Drp1-degrons leave room for improvement. For future optimization, several strategies can be tested. Based on the microscopy images, it seems that Drp1 degradation varies between cells which might be reduced in a (monoclonal) stable cell line. In addition, it was shown that degron efficiency is linked to the affinity of the binding molecule³¹¹. Therefore, bivalent or biparatopic (D7 and D63 fusion addressing two different epitopes) Drp1-degrons might lead to higher degradation efficiencies.

As Drp1 is downregulated in Alzheimer's⁵⁹, the Drp1-degrons could be applied to replicate this phenotype in disease-relevant models. In addition, in a gene therapy approach, the Drp1-degrons could be used in atherosclerosis prevention³⁹⁰ or improving cancer treatment as Drp1 overexpression was linked with a poor prognosis^{126,127}.

While we functionalized our Drp1-Nbs for application in various commonly used methods, the Drp1-Nb toolset could be further expanded. For instance, the Drp1-Nbs could be fused to both N- and C-terminal fraction of a splitFAST molecule³⁹¹ to monitor the oligomerization of Drp1.

Different aspects of Drp1 are currently investigated including its regulation³⁹²⁻³⁹⁴, role in various diseases³⁹⁵⁻³⁹⁷ and as a potential therapeutical target^{392,398,399}. Here, our Drp1-Nb toolset can facilitate these investigations due to its ability to monitor endogenous Drp1.

6.2 Discussion Miro1

Miro1 is an adaptor protein that connects mitochondria to kinesin motor proteins and is involved mitochondrial transport and mitochondrial quality control^{34,171,177,181}. In recent years, Miro1 came into focus of PD research as both diagnostic marker^{194,195} and potential therapeutic target¹⁹³. Although it is long known that mitochondrial morphology and transport are affected by ectopic expression of tagged Miro1⁴⁷, many studies still rely on this method^{181,400-404}. Thus, new tools are needed to study endogenous Miro1.

Therefore, we selected Miro1-binding Nbs, characterized them and subsequently functionalized them for applications in different research settings. To study the Miro1 interactome, Miro1-Nbs were immobilized on Sepharose beads (Miro1-traps) and subsequently applied for pulldown studies. As these monovalent traps successfully precipitated endogenous Miro1³³⁷, we asked if we could further improve them by generating bivalent Miro1-traps of the lead candidates M41 and M114. Indeed, the bivalent formats showed higher enrichment in immunoprecipitation and higher sequence coverage in MS analysis compared to their monovalent counterparts (**Figure 21**). The lack of enrichment of related Ras family proteins also indicated a high specificity of our Nbs. However, despite the significant enrichment of Miro1 for all Miro1-traps, only bivM114-trap co-precipitated class1 interactors like MFN2. In addition, known binding partners like TRAK1 and Parkin^{181,405} were not identified. This could be due to the transient nature of these interactions or steric blockage upon Nb binding. Notably, one of the TRAK1 binding sites is located between the ELMs and the C-terminal GTPase^{167,170} and both M41 and M114 bind the C-terminal GTPase domain of Miro1 (**Appendix II: Figure 3³³⁷**) resulting in a potential steric hindrance. For the previous identification of the Parkin-Miro1 interaction, the proteins were crosslinked¹⁸¹, which stabilizes transient interactions. Therefore, applying crosslinking in combination with our Miro1-traps might result in the detection of additional binding partners.

To avoid bias due to the steric hindrance of the Nb, a second Miro1-trap binding a different domain could be used complementarily. From our Miro1-Nbs, only M25 binds independently of the C-terminal GTPase domain as indicated by domain mapping (**Appendix II: Figure 3³³⁷**). While M25-trap precipitated GFP-Miro1 but not endogenous Miro1 (**Appendix II: Figure 2A, B³³⁷**), generating a bivM25-trap might increase the avidity sufficiently to enable pulldown studies with endogenous Miro1.

In conclusion, as bivM114-trap showed the highest enrichment of Miro1 as well as enrichment of known Miro1-interactors, this trap would be best suited for further experiments e.g. to study protein-protein-interactions under mitochondrial stress. By enabling the detection of endogenous Miro1, this trap is attractive for studying Miro1 in cells that are hard to transfect (e.g. neurons) and avoids bias caused by overexpression or changed protein function/interaction due to tagging. Therefore, it can replace the currently used Myc- or GST-tag-based immunoprecipitation approaches^{406,407}.

In addition, the Miro1-Nbs could be further functionalised for different research questions. For instance, the Miro1-Nbs could be combined with a Ca^{2+} -sensing FRET-based biosensor⁴⁰⁸ to further investigate how Ca^{2+} regulates Miro1 function. Thus, the Miro1-Nbs and Miro1-traps can facilitate Miro1 investigations due to their ability to monitor the endogenous protein.

6.3 Discussion cholesterol-coomassie

Successful intracellular delivery of protein-based therapeutics would substantially broaden the scope of pharmacologically addressable targets. Thus, over the years, many protein transduction methods have been introduced like different versions of cell penetrating peptides (see section 1.6). However, these are still insufficient with none reaching FDA-approval³³⁰. In 2020, Tai *et al.* published a method that combines cholesterol and coomassie, two components that are safe for human use, into a reagent for intracellular protein delivery³³⁴. With their approach, they demonstrated high transduction efficiency with low toxicity³³⁴. Therefore, I tested the method's applicability for Nb delivery.

However, I did not achieve comparable results. In my hands, the transduction efficiency was low (only up to 10 %) despite optimizing ratio and cell confluency at transduction. This transduction efficiency might be sufficient in certain experiments e.g. single cell experiments or when other delivery methods are not feasible. However, while working with low concentrations (100 nM Nb and 800 nM cho-coo), transduced cells did not divide, and nuclei were condensed indicating dead or dying cells. Therefore, either cho-coo did have toxic effects or the bivVB6_{AF488} was only able to penetrate already dying cells. Considering that bivVB6 was functional as a Cb without any major toxicity²⁵³, the cargo should not be responsible for the observed negative effects. As dying cells substantially alter their cell biology, they are inapt for investigating research questions unrelated to cell death. Thus, these results indicate that cho-coo is unsuited as a protein delivery tool as I could not replicate the effects shown by Tai *et al.* with our Nbs. It will be interesting if other groups succeed to replicate the original results.

7 Conclusions and Outlook

In this work, the previously introduced toolkit derived from of Drp1- and Miro1-binding Nbs³³⁵ was improved and extended. The two Drp1-Nbs, D7 and D63, were applied in multiple settings demonstrating their versatility and broad range of applications. Both Drp1-traps successfully precipitated endogenous Drp1 and interaction partners from various cell lysates. Functionalized into a bivalent format, bivD7 coupled to AF647 (bivD7_{AF647}) enabled the imaging of endogenous Drp1 by STORM. For live cell microscopy, D7 and D63 were formatted into turnover-accelerated Cbs enabling the monitoring of Drp1 relocalization after CCCP induction. As degrons, they enabled the inducible degradation of Drp1. However, the degradation efficiency was rather low. Therefore, the degrons could be further improved by creating bivalent or biparatopic (D7-D63 or D63-D7) versions to further improve the intracellular binding for increased proteasomal recruitment. As a bivalent format, the bivM114-trap showed improved pulldown efficiencies for endogenous Miro1 compared to the monovalent version.

Thus, these broad applications in proof-of-principle studies open the door for more advanced experiments in disease-relevant models. For instance, the Drp1-Nbs could be applied in cardiovascular disease models to study the effects of mutations or extracellular stress. Here, the Drp1-Nbs can address a broad range of research questions enabling studying the Drp1 interactome, Drp1 localization by both SRM and live cell microscopy as well as the effect of Drp1 depletion in real time.

A successful method for the intracellular delivery of recombinant protein could immensely broaden the application spectrum of Nbs. Therefore, the promising previously published cho-coo method³³⁴ was tested for their applicability. However, due to the observed toxicity and low transduction yield, this method was unsuitable. Thus, the search for a therapy-compatible protein transduction method continues.

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9 Appendix I

Nanobodies as novel tools to monitor the mitochondrial fission factor Drp1

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Methods



Nanobodies as novel tools to monitor the mitochondrial fission factor Drp1

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In cells, mitochondria undergo constant fusion and fission. An essential factor for fission is the mammalian dynamin-related protein 1 (Drp1). Dysregulation of Drp1 is associated with neurodegenerative diseases including Parkinson's, cardiovascular diseases and cancer, making Drp1 a pivotal biomarker for monitoring mitochondrial status and potential pathophysiological conditions. Here, we developed nanobodies (Nbs) as versatile binding molecules for proteomics, advanced microscopy and live cell imaging of Drp1. To specifically enrich endogenous Drp1 with interacting proteins for proteomics, we functionalized high-affinity Nbs into advanced capture matrices. Furthermore, we detected Drp1 by bivalent Nbs combined with site-directed fluorophore labelling in super-resolution STORM microscopy. For real-time imaging of Drp1, we intracellularly expressed fluorescently labelled Nbs, so-called chromobodies (Cbs). To improve the signal-to-noise ratio, we further converted Cbs into a "turnover-accelerated" format. With these imaging probes, we visualized the dynamics of endogenous Drp1 upon compound-induced mitochondrial fission in living cells. Considering the wide range of research applications, the presented Nb toolset will open up new possibilities for advanced functional studies of Drp1 in disease-relevant models.

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Introduction

Mitochondrial morphology is controlled by the balance between two opposing processes, fusion and fission. These highly regulated events adapt the cellular mitochondrial network to the bioenergetic requirements of the cell and maintain the functional integrity of mitochondria in important cellular processes such as iron

metabolism, lipid biosynthesis, calcium homeostasis and cell death (Vantaggiato et al, 2019; Iwata et al, 2020; Rasmussen et al, 2020). Mitochondrial fission in higher vertebrates is primarily mediated by the large GTPase dynamin-related protein 1 (DNM1L; Drp1 also known as DLP1). The fission process follows a coordinated sequence of events in which cytosolic Drp1 is initially recruited to mitochondrial contact sites with the ER, facilitated by ER membrane-associated actin filaments (Li et al, 2015; Ji et al, 2017; Chakrabarti et al, 2018). On the mitochondrial outer membrane (MOM), Drp1 associates with adaptor proteins including the mitochondrial dynamics protein of 49 kD (MID49, also known as MIEF2), mitochondrial dynamics protein of 51 kD (MID51; also known as MIEF1), and presumably the mitochondrial fission factor (Mff) and fission protein 1 (FIS1) (Loson et al, 2013; Palmer et al, 2013; Osellame et al, 2016). Subsequently, Drp1 oligomerizes into ring-like or helical structures surrounding mitochondria and GTP hydrolysis then triggers a conformational change in Drp1 causing the contractive division of the mitochondrial network (De Vos et al, 2005; Friedman et al, 2011; Froehlich et al, 2013; Korobova et al, 2013; Bai et al, 2015). In addition to mitochondria, Drp1 also mediates the division of peroxisomes in combination with the adaptor proteins Mff and FIS1 (Li & Gould, 2002; Koch et al, 2003; Koch & Brocard, 2012).

Dysregulation of Drp1 is associated with neurodegenerative diseases such as Alzheimer's (Cho et al, 2009; Wang et al, 2009), Huntington's (Song et al, 2011; Shirendeb et al, 2012), or Parkinson's (Han et al, 2020) as well as cardiovascular diseases (Nan et al, 2017; Jin et al, 2021). For example, immunoblotting and immunocytochemical analyses revealed reduced Drp1 protein levels in hippocampal tissues of Alzheimer's patients compared with age-matched healthy individuals (Wang et al, 2009), whereas Huntington's disease patients were found to have increased Drp1 enzymatic activity mediated by interaction with mutant Huntingtin (Htt) aggregates, resulting in abnormal mitochondrial dynamics, including defective anterograde mitochondrial movement and

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synaptic deficiencies (Song et al, 2011; Shirendeb et al, 2012). More recently immunoblotting revealed lower phosphorylation levels of Drp1 at serine 616 (S616) in dermal fibroblasts from Parkinson's patients compared with healthy individuals (Han et al, 2020). In addition, elevated Drp1 expression and phosphorylation on S616 detected by immunoblotting and immunocytochemical analyses have been linked to cancer, including melanoma, glioblastoma, lung, breast, thyroid, pancreatic, and head and neck cancer (Rehman et al, 2012; Zhao et al, 2013; Ferreira-da-Silva et al, 2015; Kashatus et al, 2015; Serasinghe et al, 2015; Liang et al, 2020; Huang et al, 2022). It is assumed that an increased Drp1 level promotes mitochondrial cleavage and thus aerobic glycolysis, thereby promoting the growth and metastasis of tumor cells (Liang et al, 2020). In this context, knockdown of Drp1 was shown to be associated with a marked reduction in cancer cell proliferation and an increase in spontaneous apoptosis (Rehman et al, 2012; Kashatus et al, 2015; Liang et al, 2020). These findings highlight the growing importance of Drp1 as a biomarker or potential target for therapeutic interventions. However, despite several in vitro and in vivo studies, detailed information on the molecular mechanism and structural changes of Drp1 during mitochondrial fission, as well as its cellular dynamics and interacting components, is still lacking (Giacomello et al, 2020; Tong et al, 2020; Zerihun et al, 2023). This gap in knowledge is partly because of the limited availability of research tools to study Drp1. Notably, most live cell analyses rely on ectopic expression of fluorescent fusion constructs or epitope-tagged Drp1 (Labrousse et al, 1999; Solesio et al, 2013; Ji et al, 2017; Michalska et al, 2018; Xiong et al, 2022). However, recent studies revealed that N- or C-terminal labelling of Drp1 with fluorescent proteins or epitope tags leads to altered oligomerization dynamics and impairs its GTPase activity (Montecinos-Franjola et al, 2020).

Single-domain antibody fragments derived from the heavy chain-only antibodies of camelids (Hamers-Casterman et al, 1993), also known as nanobodies (Nbs), have emerged as versatile research tools in biomedical research. Because of their specific binding properties, small size, high stability, and solubility, these binders became an attractive alternative to conventional antibodies in many biochemical and cell biological research applications (Frecot et al, 2023). In addition, intracellular functional Nbs genetically fused to fluorescent proteins, so-called chromobodies (Cbs), serve as imaging probes to visualize dynamic changes of target antigens without genetic or covalent modifications and can be applied in different cellular compartments as well as in whole organisms (Wagner & Rothbauer, 2020). Here, we have identified a set of novel Nbs specifically recognizing human Drp1. After their detailed biochemical, biophysical and functional characterization, we have developed them as broadly applicable research tools. Thus, we converted them into affinity matrices for proteomic analysis of Drp1 and developed bivalent Nbs as labelling probes to detect endogenous Drp1 in confocal and super-resolution microscopy (SRM). By engineering turnover-accelerated Drp1-Cbs, which have an improved signal-to-noise ratio, we were able to visualize the drug-induced recruitment of Drp1 to mitochondria in living cells. Considering the broad spectrum of applications, we propose that the Drp1-specific Nbs/Cbs described herein open up new possibilities for future advanced functional studies of Drp1 in

disease-relevant models and represent a promising alternative to currently available approaches.

Results

Identification of Drp1-specific Nbs

To generate Nbs against human Drp1 (Drp1), we immunized an alpaca (*Vicugna pacos*) with recombinant Drp1 using a 91-d immunization protocol and detected a specific immune response in a serum ELISA 63 d after the first vaccination (Fig S1A). Subsequently, we established an Nb phagemid library (size $\sim 2 \times 10^7$ clones) from the mRNA of the peripheral B lymphocytes, which was subjected to phage display using either passively adsorbed or biotinylated Drp1. After two rounds of biopanning, we analysed 260 individual clones in a phage ELISA and identified 27 positive binders (Fig S1B) with eight unique sequences (Table S1). Notably, only two Nbs, D7 and D63, displaying highly diverse complementarity determining regions 3 (Fig 1A), showed sufficient binding signals in a protein ELISA using periplasmic extracts derived from Nb-expressing *Escherichia coli* (*E. coli*) (Fig S1C). Therefore, we purified these binders by immobilized metal affinity chromatography and subsequent size exclusion chromatography (Fig 1B). For determining their binding affinities, both Nbs were subjected to biolayer interferometry. Biotinylated Nbs were immobilized on streptavidin (SA) biosensors and binding kinetics were measured by titrating varying concentrations of Drp1. Both Nbs, D7 and D63, showed affinities in the low nanomolar range with K_D values of ~ 3.5 and ~ 1.8 nM, respectively (Figs 1C and S2). In addition, analysing their folding stability using nano-differential scanning fluorimetry (NanoDSF) revealed that both Nbs exhibit stable folding and a low tendency to aggregate having melting temperatures of ~ 68 and $\sim 70^\circ\text{C}$ for D7 and D63, respectively (Fig 1D). To further assess whether Nb binding affects the functionality of Drp1, we measured the GTPase activity of Drp1 in the presence of the respective Nbs in vitro. Whereas D7 and the negative control (GFP Nb) had no effect on the enzymatic activity of Drp1, we observed a significant increase in the GTPase rate by a factor of ~ 1.7 after addition of a 10-fold molar excess of D63 (Fig 1E).

Selected Drp1-Nbs recognize different domains of Drp1 and precipitate endogenous Drp1

Considering that Drp1 is composed of multiple domains including an N-terminal GTPase (aa 1–337), an unfolded (middle, stalk, aa 338–502), a variable (B insert, aa 503–635), and a C-terminal GTPase effector domain (GED) region (aa 636–736) (Otera et al, 2013), we constructed a series of GFP-labelled domain deletions of Drp1 (Fig 2A) and tested whether the selected Nbs recognize specific domains within Drp1 by pull-down assays. To this end, we generated so-called Drp1 nanotraps, for which we covalently immobilized D7 and D63 on N-hydroxysuccinimide (NHS)-activated sepharose beads via primary amino groups of accessible lysine residues (Traenkle et al, 2020; Fagbadebo et al, 2022). For binding analysis, the Drp1 nanotraps were incubated with soluble protein fractions of HEK293 cells transiently expressing the Drp1 deletion constructs.

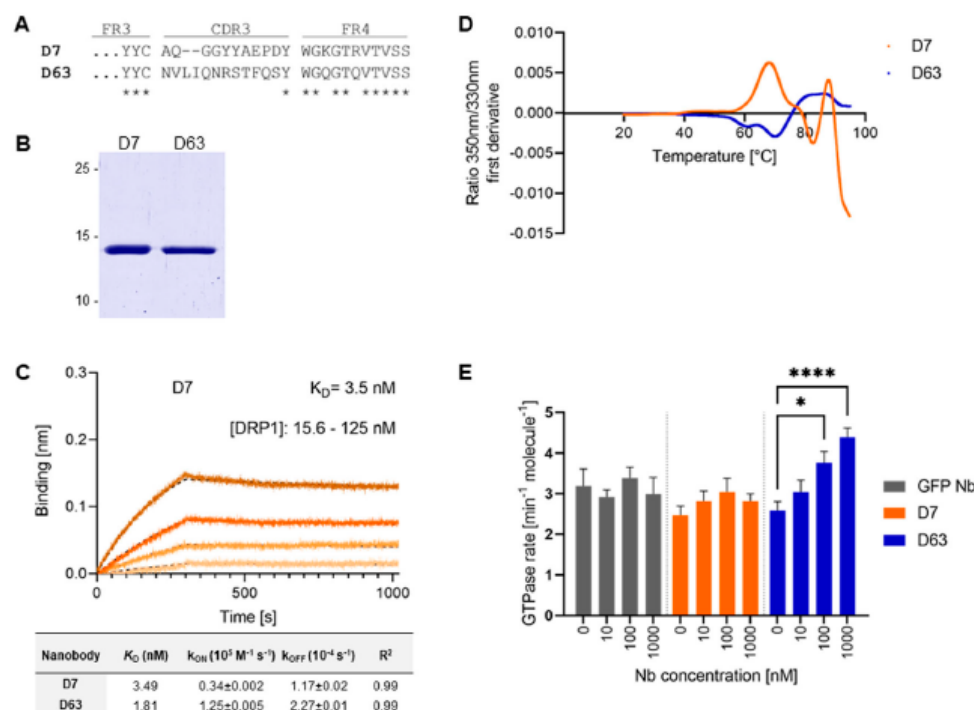


Figure 1. Identification and biochemical characterization of Drp1-specific nanobodies.

(A) Amino acid (aa) sequences of the complementary determining region 3 (CDR3) of the nanobodies (Nbs) D7 and D63 identified by protein ELISA. (B) Recombinant expression and purification of D7 and D63 by immobilized metal affinity chromatography and size exclusion chromatography. Coomassie staining of purified Nbs (2 μ g) is shown. (C) Biolayer interferometry-based affinity measurements exemplarily shown for D7. Biotinylated Nb D7 was immobilized on streptavidin sensors. Kinetic measurements were performed using four concentrations of purified Drp1 ranging from 15.6–125 nM (displayed with gradually darker shades of color). The binding affinity (K_D) was calculated from global 1:1 fit shown as dashed lines. Affinities (K_D), association constants (k_{on}), and dissociation constants (k_{off}) of D7 and D63 determined by BLI shown as mean $\pm$ SD. (D) Stability analysis by nano-differential scanning fluorimetry displaying the fluorescence ratio (350/330 nm) first derivative for D7 (orange) and D63 (blue). Data are shown as the mean value of three technical replicates. (E) GTP hydrolysis of 100 nM Drp1 in solution in the presence of increasing concentrations of different Nbs at 37°C (mean $\pm$ SED, $n = 5$ experiments on 4 d). Significance was tested using two-way ANOVA and multiple comparison analysis in GraphPad Prism (n.s. $P > 0.05$, * $P < 0.05$, *** $P < 0.0001$).

Here we used the GFP-Trap as a positive control (Rothbauer et al, 2008). Immunoblot analysis revealed that both Nbs precipitated full-length Drp1 as expected (Fig 2B). However, whereas D7 exclusively recognizes the N-terminal GTPase domain, D63 only binds in the presence of the GED, whereas none of the Nbs binds non-specifically to GFP (Fig 2B). Next, we tested the capacity of the Drp1 nanotraps to capture endogenous Drp1. Therefore, we generated soluble protein extracts from three different human cell lines (HEK293, HeLa, and U2OS) and incubated them with the Drp1 nanotraps. For comparison, we used a monoclonal anti-Drp1 antibody immobilized on proteinA/G-sepharose as positive control (PC) and the GFP-Trap as negative control (NC). Immunoblot analysis of all cell lines tested showed that both Drp1 nanotraps precipitated higher levels of endogenous Drp1 compared with the conventional antibody (Figs 3A and S3A and B).

For a more detailed insight, we analysed the proteome of both Drp1 nanotraps by mass spectrometry (MS) derived from the soluble protein fractions of HEK293, HeLa and U2OS cells. Immunoprecipitations were performed for all cell lines in three replicates,

each with the same number of cells as starting material. As exemplarily shown for HEK293 cells, the results were highly reproducible showing a Spearman rank correlations close to one (Fig S4A). As expected, the correlation between the two different Drp1 nanotraps was higher than between each nanotrap and the control (GFP-Trap), confirmed by principal component analysis (Fig S4B). We next evaluated how well each nanotrap captured endogenous Drp1. Both nanotraps enabled the identification of multiple Drp1 peptides comparably, resulting in high coverage of the Drp1 sequence (~70%) (Fig S4C). Notably, 55 “Razor” peptides matching Drp1 isoform 1, 5, 6, or 7 were found in the precipitates of both nanotraps (Fig S4D). However, based on our dataset, we could not distinguish between these isoforms. Next, we confirmed the efficacy of Drp1 enrichment. Overall, the number of protein groups (PGs) identified was comparable for all samples, with a background of up to 981 proteins. For both nanotraps, Drp1 was one of the most abundant proteins detected, whereas it was not identified in the negative control (Fig 3B). Furthermore, Drp1 was the most significantly enriched protein with both traps (Fig 3C and D). Finally, we scanned

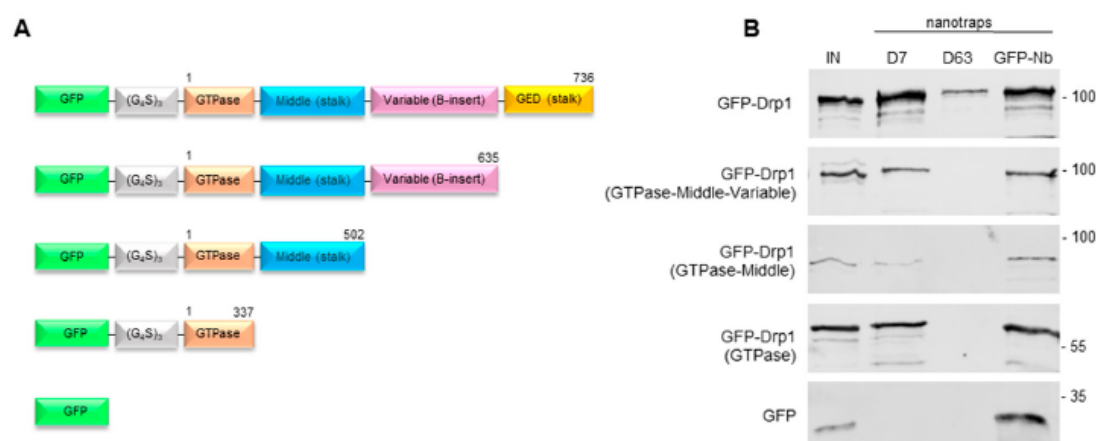


Figure 2. Domain mapping of Drp1-Nbs.

(A) Schematic illustration of the GFP-labelled Drp1 deletion constructs used for domain binding studies. Numbers indicate amino acid positions of the Drp1 coding sequence. (B) Results of the pull-down analysis of the individual Drp1 deletions as depicted in (A) using the Drp1-specific nanotraps (D7, D63) or the GFP-Trap (GFP-Nb) as positive control. Input (IN, 1% of total) and bound (20% of total) fractions were subjected to SDS-PAGE followed by immunoblot analysis using an anti-GFP antibody. Molecular weights in kD are indicated on the right.

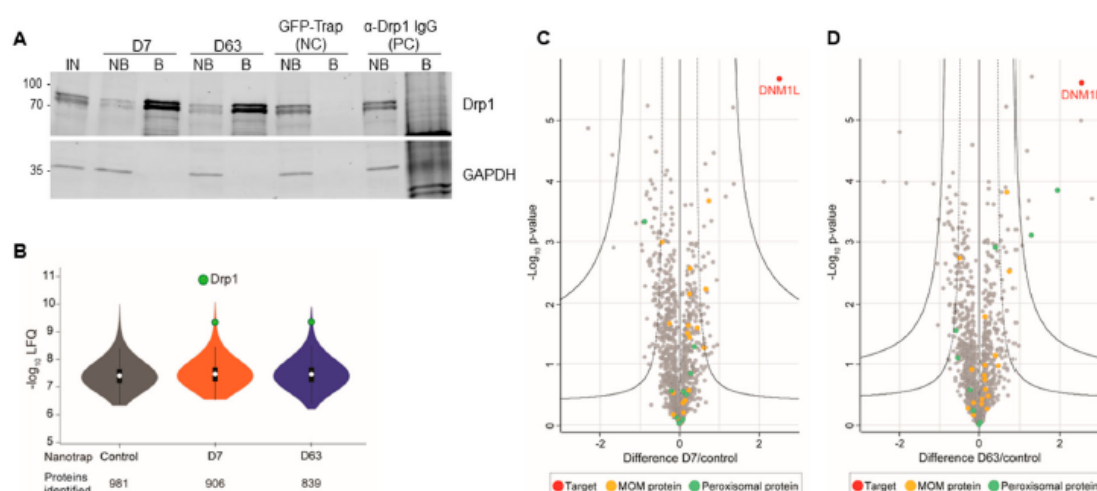


Figure 3. Immunoprecipitation of endogenous Drp1 derived from HEK293 cells with mass spectrometry analysis of the interactome of the Drp1 nanotraps.

(A) Representative result of an immunoprecipitation of endogenous Drp1 with Drp1-specific nanotraps (D7 and D63) using soluble protein fraction of HEK293 cells. As negative control (NC), the GFP-Trap as non-specific nanotrap was used and bead-coupled anti-Drp1 IgG served as a positive control (PC). Input (IN, 1% of total), non-bound (NB, 1% of total) and bound (B, 33% of total) fractions were subjected to SDS-PAGE followed by immunoblot analysis using antibodies specific for Drp1 (upper panel) and GAPDH (lower panel). Molecular weights in kD are indicated on the left. (B) Violin plot of the averaged label-free quantification intensities of the proteins identified from immunoprecipitation using HEK cells. White circles show the median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; polygons represent density estimates of data and extend to extreme values. Drp1 is marked in green. (C, D) Multi-volcano analysis (Hawaiian plot) of the pull-down of D7 (C) and D63 (D) against the negative control. Log₂ transformed ratios of the difference between the Drp1 nanotrap and the control (x axis) are plotted against the log₁₀ transformed P-value (y axis). Drp1 is marked in red, mitochondrial outer membrane proteins are marked in yellow and peroxisomal proteins in green. Significant interactors can be class A hits (higher confidence, $s_0 = 0.1$, FDR = 0.01) or class B hits (lower confidence, $s_0 = 0.1$, FDR = 0.05), thresholds are displayed as a solid line and a dashed line, respectively.

our MS data for potential interaction partners of Drp1. Interestingly, both Drp1 nanotraps facilitated the enrichment of cytoplasmic or mitochondrial interactors compared with the control, whereas only the D63 nanotraps allowed for the enrichment of class A interactors

of peroxisomal proteins such as HSDL2 (hydroxysteroid dehydrogenase-like protein 2) or HSD17B4 (peroxisomal multifunctional enzyme type 2) (Fig 3C and D; Tables S2 and S3). In addition, with both Drp1 nanotraps, we found a significant

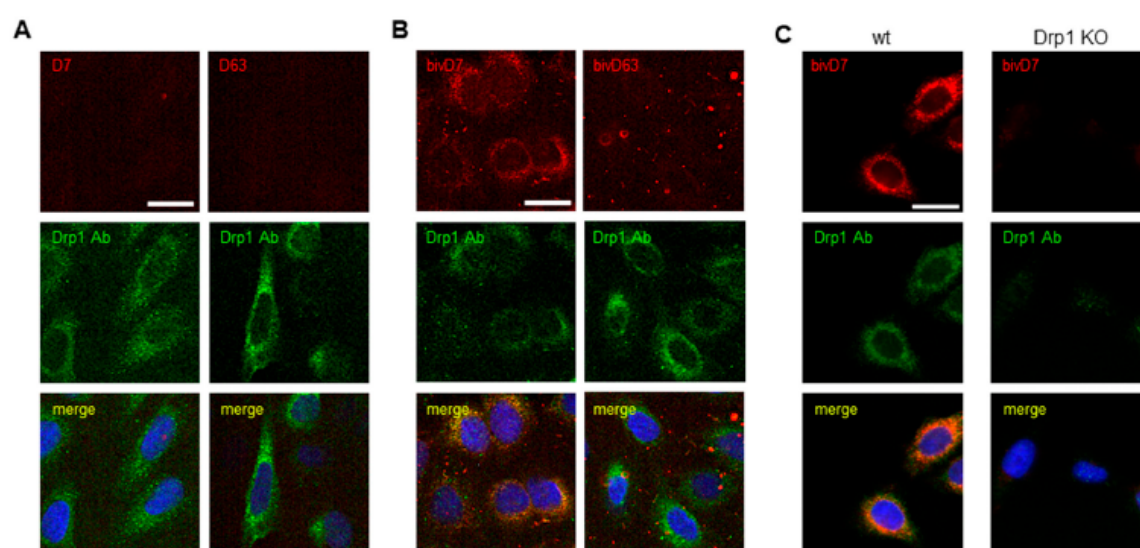


Figure 4. Immunofluorescence staining with Drp1-Nbs.

(A) Representative confocal laser scanning microscopy images of U2OS cells stained with D7 and D63 Nbs as primary labelling probes detected with an anti-VHH antibody labelled with Cy5. As positive control, cells were co-stained with anti-Drp1 antibody (Drp1 Ab) followed by detection with an AlexaFluor488-labelled secondary antibody. (B) Immunofluorescence (IF) detection of Drp1 in fixed and permeabilized U2OS cells after staining with bivalent versions of D7 and D63 (bivD7, bivD63) as described in (A). (C) Immunofluorescence (IF) detection of Drp1 in fixed and permeabilized wt HeLa (left column) and Drp1 KO HeLa cells (right column) stained with bivD7 as described in (B). (A, B, C) Nuclei were counterstained with DAPI. Scale bar 25 μ m.

enrichment of CDGSH iron-sulfur domain-containing protein 1 (CISD1) and Histone deacetylase 6 (HDAC6), two factors previously reported to interact with Drp1 (English & Barton, 2021; Hua et al, 2021) (Tables S2 and S3).

Notably, both Drp1 nanotraps performed similarly in immunoprecipitation of Drp1 when using HeLa and U2OS cells and proteomic analysis of the bound fraction yielded comparable results (Figs S5A–E and S6A–E). Interestingly, we observed an additional enrichment of isoform 2 of Drp1 in HeLa cells with both nanotraps (Fig S5F and G), whereas in U2OS cells, both nanotraps also precipitated isoforms 4 or 8 (Fig S6F and G). These results are likely because of the expression of different isoforms of Drp1 in these cell lines. Furthermore, we observed a slightly different enrichment of potential interaction partners of Drp1 (Tables S4 and S5).

In summary, these results showed that the selected Nbs recognize different domains of Drp1 and can be readily converted into functional capture reagents to precipitate endogenous Drp1 together with its interaction partners.

bivD7 enables immunofluorescence detection of endogenous Drp1

To further examine the potential of the Drp1-Nbs as research tools, we investigated their performance in immunofluorescence (IF) microscopy. First, we applied D7 and D63 as primary binding molecules in combination with a fluorescently labelled anti-VHH antibody in fixed and permeabilized U2OS cells transiently overexpressing GFP-Drp1. Only D7 showed colocalization with GFP-Drp1,

whereas D63 showed no staining (Fig S7), suggesting that this Nb does not bind Drp1 in IF. Furthermore, when we tested IF for endogenous Drp1 in U2OS cells, we did not observe specific Drp1 staining for either Nb (Fig 4A). Considering that a bivalent format can be superior to monovalent versions for imaging purposes (Virant et al, 2018; Fagbadebo et al, 2022), we genetically fused two D7 or two D63 Nbs head-to-tail connected by a flexible Gly-Ser linker [(G₄S)₄] and generated a bivalent format of D7 (bivD7) or D63 (bivD63), respectively. In addition, we inserted a sortase-tag for site-directed labelling. Both bivalent Nbs were purified as secreted proteins from ExpiCHO cells (Fig S8A) followed by measuring their binding affinities as described for the monovalent versions. The results showed that bivD7 had an increased affinity in the sub-nanomolar range due to decelerated dissociation, whereas the affinity of bivD63 did not increase compared with the monovalent version (Fig S8B and C). Initially, we tested both bivalent Nbs for colocalization with GFP-Drp1 in U2OS cells and observed more specific signals compared with the monovalent versions, with bivD7 tending to recognize Drp1 more potently compared with bivD63 (Fig S9). Next, we applied bivD7 and bivD63 for staining of endogenous Drp1. Notably, only the bivD7 showed a clear colocalization with the antibody-labelled Drp1 in U2OS cells (Fig 4B). To further confirm that bivD7 specifically recognizes Drp1, we performed immunofluorescence (IF) imaging of wt and Drp1 KO HeLa cells. Whereas a colocalization with the Drp1 antibody signal was detected in wt HeLa cells, no Nb-derived signal was observed in Drp1 KO HeLa cells (Figs 4C and S10A and B). From these results, we concluded that the bivalent format of D7 is a suitable probe for IF applications to visualize endogenous Drp1.

bivD7 is suitable for SRM

Because of their small size and their ability to access dense cell compartments and structures, Nbs have been previously described as highly versatile probes for SRM (Koch & Brocard, 2012; Virant et al, 2018; Cramer et al, 2019; Götzke et al, 2019; Maidorn et al, 2019; Driouchi et al, 2022). Hence, we tested the bivD7 in SRM using stochastic optical reconstruction microscopy (STORM). For site-directed labelling, we introduced a peptide comprising an azide group at the C-terminus by chemoenzymatic sortagging (Popp & Ploegh, 2011; Virant et al, 2018), followed by addition of a dibenzocyclooctyne (DBCO) derivative by click chemistry (Fagbadebo et al, 2022) which allowed us to flexibly and specifically conjugate STORM-compatible dyes such as AlexaFluor 647 (AF647). For initial validation, we stained wt HeLa and Drp1 KO HeLa cells with the fluorescently labelled bivD7_{AF647}, confirming its functionality as an imaging probe (Fig S11). In addition, we stained Drp1 in a cell line expressing monomeric enhanced GFP (mEGFP)-tagged Drp1 (mEGFP-Drp1) at endogenous expression levels. Confocal imaging and colocalization analysis revealed a reasonable overlap of the mEGFP-Drp1 and the bivD7_{AF647} signal with a Pearson's correlation coefficient of 0.73 ± 0.07 (Fig 5A–C). Next, we imaged Drp1 stained with bivD7_{AF647} in mEGFP-Drp1 U2OS cells using STORM (Fig 5D). The reconstituted bivD7_{AF647} signal overlaid with the epifluorescence signal of mEGFP-Drp1 acquired in the same cell before STORM imaging as expected. These results demonstrate the applicability of the bivD7_{AF647} as an imaging probe with enough brightness and specificity to label Drp1 at endogenous expression levels. Finally, we imaged bivD7_{AF647}-stained Drp1 in wt U2OS cells by STORM. Here, we detected localizations of both diffuse cytosolic Drp1 as well as mitochondrial Drp1 complexes (Fig 5E and F). Utilizing the bivD7_{AF647} for STORM imaging allowed us to resolve macromolecular assemblies of Drp1 at mitochondria demonstrating its usability as a specific Drp1 probe for SRM imaging with minimal linkage error (Fig 5G).

Drp1-Cbs visualize the dynamics of endogenous Drp1 in live cells

To further visualize endogenous Drp1 in a “tag-free” approach in living cells, we generated Cb expression constructs by genetic fusion of D7 and D63 with TagRFP via a flexible Gly-Ser linker (Fig 6A). First, we tested the intracellular binding properties of the newly generated Drp1-Cbs by intracellular immunoprecipitation (IC-IP) (Maier et al, 2015; Traenkle et al, 2015). To this end, we transiently transfected HEK293 cells with the Drp1-Cbs or an unrelated Cb (Pep Cb, [Traenkle et al, 2020]) as a negative control (NC), precipitated the Cbs with a RFP-Trap, and analysed the bound fraction for endogenous Drp1. Immunoblot analysis showed enrichment of endogenous Drp1 along with the precipitated Drp1-Cbs, but not with the negative control (Fig 6A), from which we concluded that both Drp1-Cbs retain their binding properties upon intracellular expression. When using Cbs as intracellular probes in general, it is important to be aware that the fluorescent emission signal originating from the unbound Cbs may mask the signals of the antigen-bound Cbs. This is particularly a problem for the detection of Drp1, as Drp1 is predominantly localized in the cytosol and

unbound cytosolic Cbs result in a high background. This limits the specific detection of Drp1 and, for example, its recruitment to mitochondrial fission sites. To improve the signal-to-noise ratio, we further modified our Drp1-Cbs and generated turnover-accelerated versions that are subject to faster proteasomal degradation when unbound, as previously described (Keller et al, 2018). We tested the applicability of this approach by expressing both standard and turnover-accelerated D7 and D63 Cbs in wt HeLa and Drp1 KO HeLa cells. Quantitative imaging of living cells using nuclear-localized GFP for transfection control and cell segmentation revealed significantly lower average cellular intensities and thus a lower cytosolic background originating from the unbound Cb fraction in the case of turnover-accelerated Cbs compared with their unmodified counterpart (Fig 6B and C). Moreover, in Drp1 KO HeLa cells the signal of turnover-accelerated Cbs was further reduced because of the lack of antigen (Fig 6B and C). This decrease indicates that the modification significantly reduces the amount of unbound Drp1-Cbs and increases the signal-to-noise ratio of these intracellular imaging probes. Finally, we analysed the potency of turnover-accelerated Drp1-Cbs to visualize the dynamic relocation of Drp1 from the cytosol to the mitochondria after chemical induction of mitochondrial fission. To induce fission and thus, recruitment of Drp1 to mitochondria, we treated U2OS cells transiently expressing unmodified or turnover-accelerated Drp1-Cbs with carbonyl cyanide m-chlorophenylhydrazone (CCCP), an uncoupler of mitochondrial oxidative phosphorylation. CCCP was previously reported to induce fission and Drp1 translocation to mitochondria (Voccoli & Colombaioni, 2009; Jones et al, 2017; Park et al, 2018; Pascucci et al, 2021). Time-lapse imaging of cells expressing the unmodified Drp1-Cbs showed rather diffuse Cb signals that did not change over time. In contrast, in cells expressing the turnover-accelerated Drp1-Cbs, an increasing number of spots became visible within the observation period (Fig 7), which was not observable in the absence of CCCP (Fig S12). Based on the colocalization with the MitoTracker signal, these characteristic spots strongly suggest an accumulation of Drp1 at mitochondrial fission sites. Notably, we did not observe such patterns in Drp1 KO HeLa cells, which further underlines that the detected spots are most likely Drp1 complexes bound by the Drp1-Cbs (Fig S13). In summary, these findings indicated that turnover-accelerated Drp1-specific Cbs are suitable for monitoring and visualizing the dynamic localization of endogenous Drp1 at the MOM after induction of mitochondrial fission in living cells.

Discussion

There is increasing evidence of dysregulated Drp1 in the context of a variety of diseases, including neurodegeneration such as Parkinson's (Han et al, 2020), cardiovascular diseases (Zunino et al, 2007; Sharp et al, 2014; Kim et al, 2015) and cancer (Rehman et al, 2012; Kashatus et al, 2015; Serasinghe et al, 2015). However, the exact molecular mechanisms of Drp1 function and its dynamic regulation remain to be elucidated. Therefore, the development of advanced research tools to study this key player in

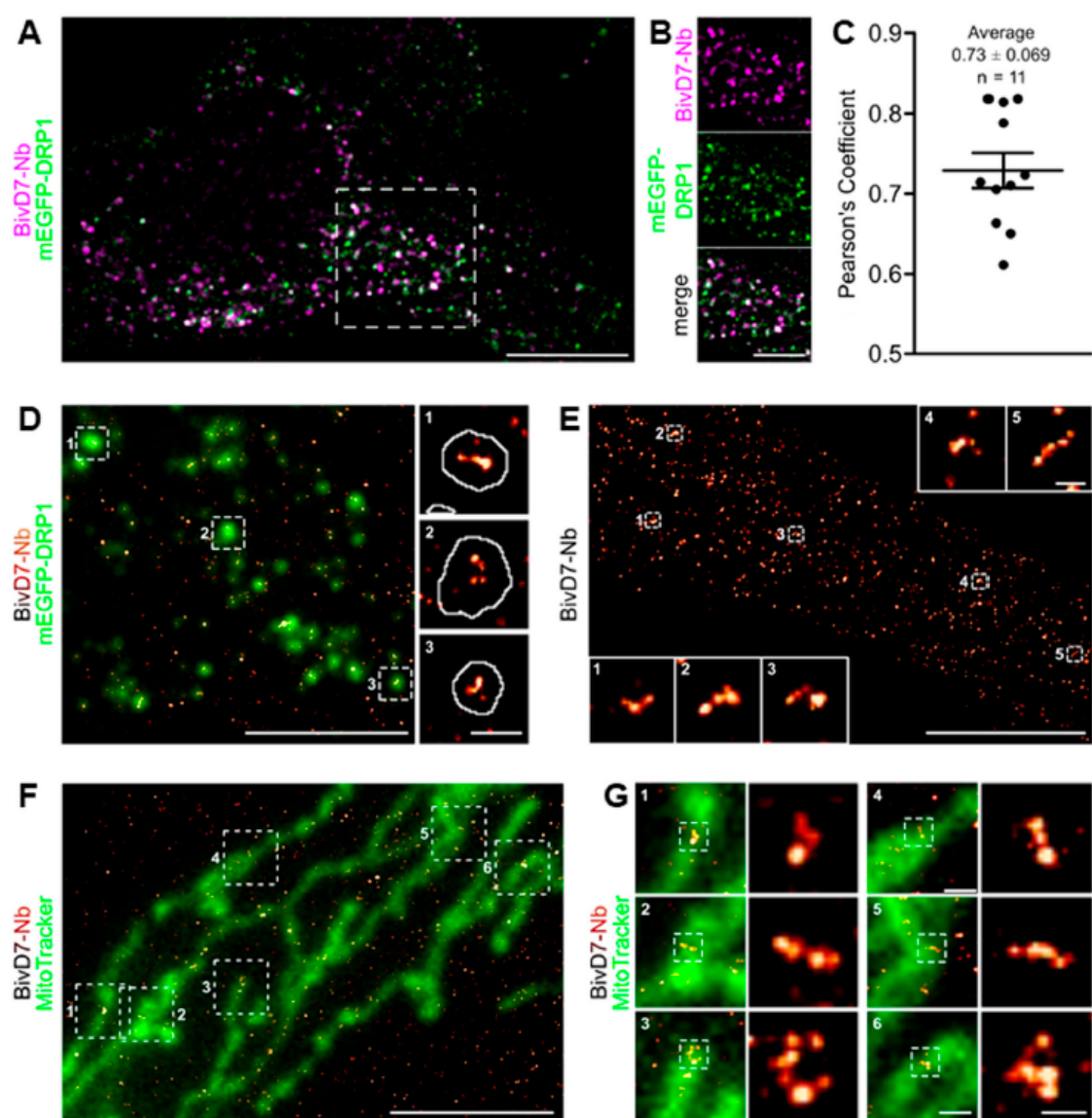


Figure 5. Super-resolution imaging of Drp1 in U2OS cells.

(A) Representative confocal fluorescence microscopy image of U2OS mEGFP-Drp1 cells (mEGFP-Drp1 signal shown in green) stained with bivD7 directly conjugated to AlexaFluor647 (bivD7_{Alexa647}; magenta). (B) Zoomed images correspond to crop regions as indicated in (A). Scale bar 10 μ m, crop 5 μ m. (C) Pearson's correlation coefficient of mEGFP and bivD7_{Alexa647} fluorescence emission signals calculated from background-corrected images shown in (A). Data are representative for $n = 3$ independent experiments with $n = 11$ cells. (D) Reconstructed super-resolution image of Drp1 stained with bivD7_{Alexa647} (orange-hot) overlaid with the epifluorescence signal of mEGFP-Drp1 (green). Zoomed images (right) correspond to cropped regions as indicated. White line in the zoomed regions corresponds to the outline of the thresholded mEGFP-Drp1 signal. Images are representative for $n = 3$ independent experiments. Scale bar 5 μ m, zoom 500 nm. (E) Reconstructed super-resolution image of Drp1 labelled with bivD7_{Alexa647} in wt U2OS cells. Insets correspond to zoomed regions as indicated. Scale bar 5 μ m, insets 200 nm. (F) Reconstructed super-resolution image of Drp1 labelled with bivD7_{Alexa647} (orange-hot) overlaid with an epifluorescence microscopy image of mitochondria stained with MitoTracker (green) in wt U2OS cells. Scale bar 5 μ m. (G) Zoomed areas of the image shown in (F) (left images) with further zoom on Drp1 assemblies (right images) as indicated. Scale bar 500 nm, zoomed images 200 nm. (D, E, F, G) Data are representative of at least $n = 3$ independent experiments.

mitochondrial fission is urgently needed (Giacomello et al, 2020; Montecinos-Franjola et al, 2020; Tong et al, 2020; Zerihun et al, 2023).

Here, we present, to the best of our knowledge for the first time, an Nb-based toolset for studying endogenous Drp1 in different biochemical and cell biological research settings. We identified two

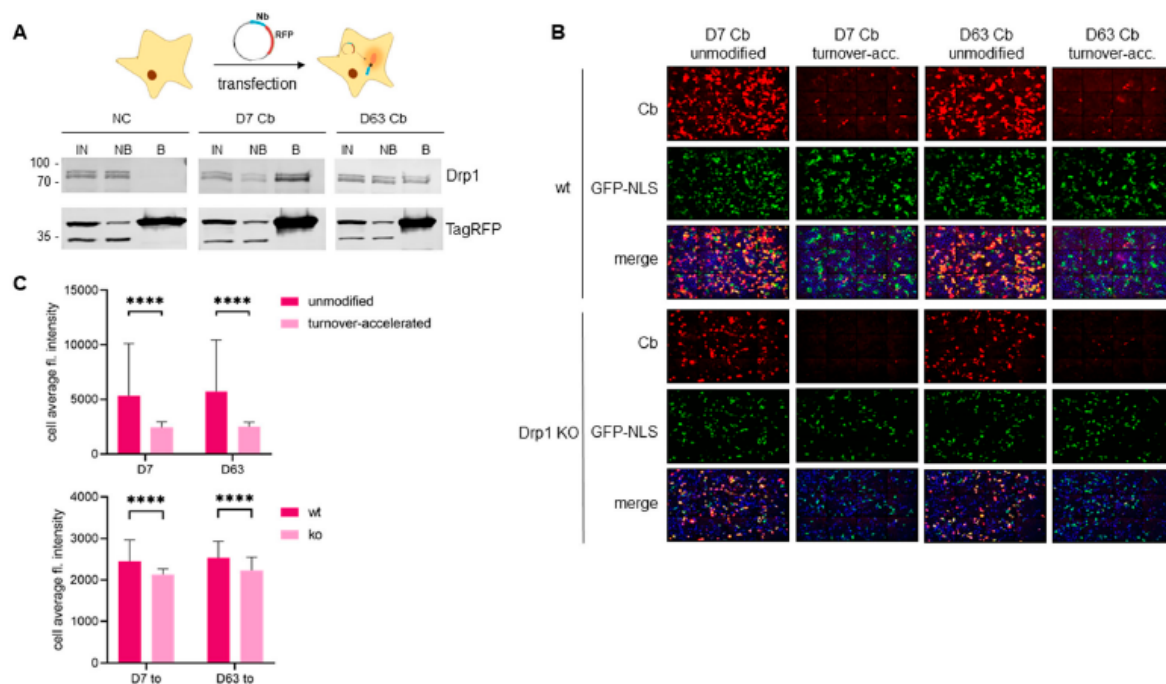


Figure 6. D7 and D63 Cb recognize their antigen and are stabilized in the presence of Drp1 in living cells.

(A) Intracellular immunoprecipitation of Drp1. Soluble protein fractions of HEK293 cells expressing the indicated chromobodies (D7 Cb, D63 Cb or as negative control an unrelated chromobody [NC]) were subjected to immunoprecipitation with the RFP-Trap. Input (IN), non-bound (NB) and bound fractions (B) were analysed by immunoblotting with an anti-Drp1 antibody (upper panel) and an anti-TagRFP antibody (lower panel). Molecular weights in kD on the left. (B) Representative fluorescence images of living wt HeLa or Drp1 KO HeLa cells transiently expressing either the unmodified or the turnover-accelerated Drp1-Cb constructs. As transfection control, a GFP-NLS encoding construct was co-transfected. Nuclei were stained with Hoechst33258. (C) Bar chart representing the cell average fluorescence intensity of Cb signal (red channel) quantified from (B). For the comparison of unmodified and turnover-accelerated Cbs, significance was calculated using Kruskal Wallis test with Dunn's multiple comparisons test (upper panel). For the comparison of the turnover-accelerated Cb (D7 to; D63 to) intensities in wt HeLa cells and Drp1 KO cells, the significance was calculated using two-way ANOVA with Sidak's multiple comparisons (lower panel). Data are presented as mean $\pm$ SD; n.s. $P > 0.05$, **** $P < 0.0001$; $n > 130$ cells each.

stable and well-producible Nbs, D7 and D63, which bind distinct domains of Drp1 with high affinity. Notably, binding of D63 to the C-terminal GED of Drp1 induces an increased GTPase activity in vitro. It can be speculated that this is because of a stabilization of the intramolecular interaction between the GED and the N-terminal GTP-binding domain—a conformation known to be important for the enzymatic function of Drp1 (Zhu et al, 2004; Frohlich et al, 2013). By targeted functionalization and engineering, we have successfully applied the identified Nbs as (i) affinity capture tools (Drp1 nanotraps), (ii) labelling probes for fluorescence microscopy including SRM, and (iii) Cbs for visualization of Drp1 in living cells. For affinity capture from whole cell lysates, we demonstrated that both Drp1 nanotraps efficiently precipitated Drp1. Notably, immunoblot analysis with a monoclonal Drp1 antibody showed captured Drp1 in the form of several bands. These findings are comparable with previous reports also showing multiple bands for Drp1 (Loson et al, 2013; Yu et al, 2019; Xie et al, 2020; Yu et al, 2021), which can be attributed to the size differences between individual Drp1 isoforms (Chen et al, 2000; Itoh et al, 2018). Applying the nanotraps to precipitate Drp1 from other cell types or even tissues, might result

in the identification of a different Drp1 pattern reflecting the expression of different isoforms. Indeed, we observed an enrichment of different Drp1 isoforms when we used the Drp1 nanotraps for immunoprecipitation studies in different cell lines. With in-depth MS analysis of the proteome of both Drp1 nanotraps derived from the soluble fraction of three different cell lines, we confirmed the effective and specific enrichment of Drp1 with a high sequence coverage based on the identified peptides. It is noteworthy that the MS analysis also revealed the enrichment of MOM- and peroxisome-associated factors. Interestingly, the latter were found exclusively for the D63 nanotrap, but not for the D7 nanotrap, which could be explained by steric and allosteric effects caused by the binding of the Nbs at the different domains of Drp1. Overall, both Drp1 nanotraps precipitated endogenous Drp1 more efficiently than the conventional monoclonal Drp1 antibody. Thus, we conceive that both Nb-based affinity matrices enable a fast and efficient investigation of the Drp1-interatome under different physiological conditions and/or in different tissues.

Despite their high affinity for recombinant Drp1, both Nbs initially failed to stain endogenous Drp1 by immunofluorescence labelling.

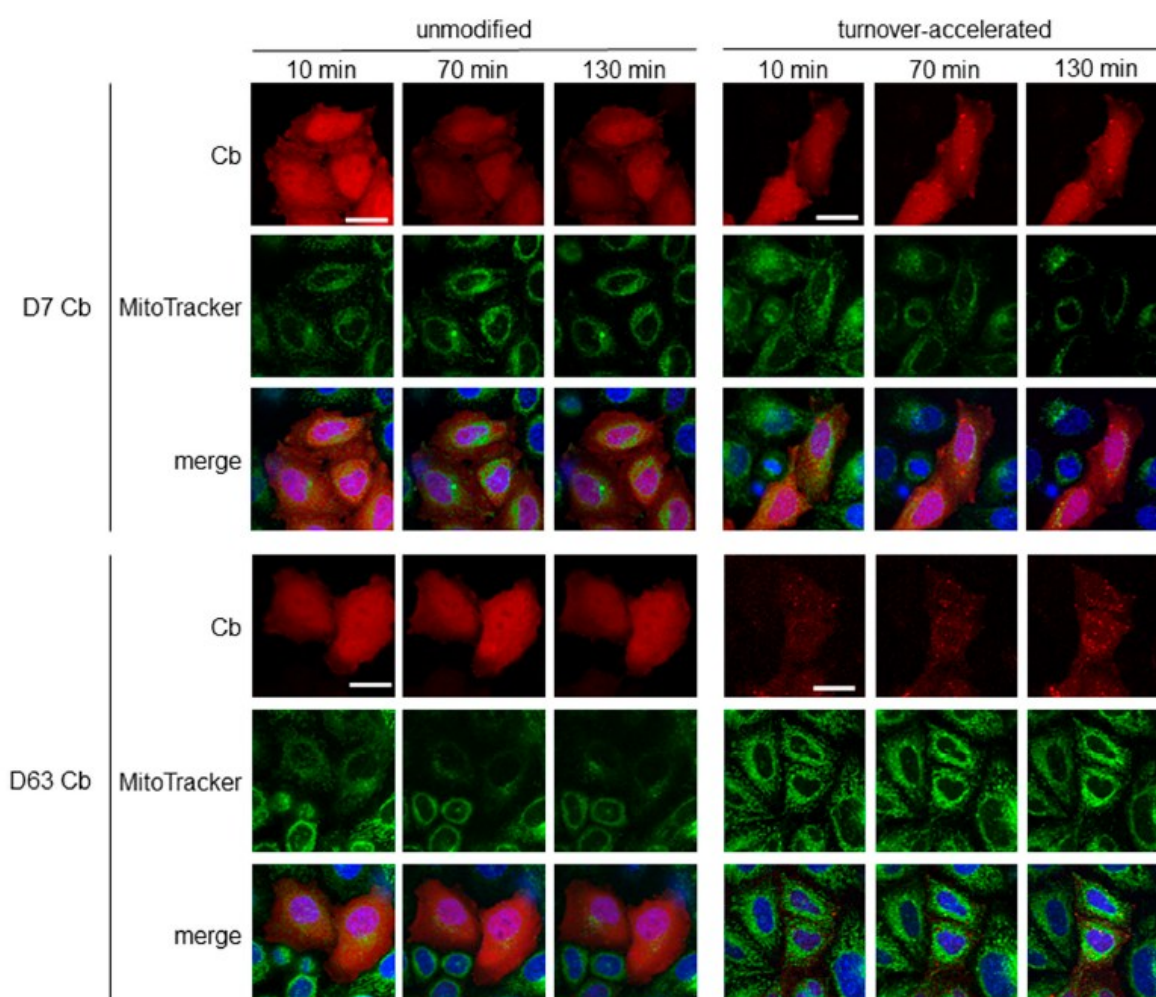


Figure 7. Turnover-accelerated Cbs monitor relocation of Drp1 in living cells. Fluorescent time-lapse microscopy of wt HeLa cells transiently expressing unmodified or turnover-accelerated chromobodies (Cbs) (red). 24 h post transfection, cells were stained with Hoechst33258 (blue) and MitoTracker green (green) and subsequently treated with 20 μ M cyanide m-chlorophenylhydrazine (CCCP). Representative confocal images were taken after 10, 70, and 130 min of treatment. For each Cb, brightness and contrast were individually set and kept for all time points. Scale bar 25 μ m.

This might be because a lower accessibility of the epitope or a higher off-rate of the Nbs for Drp1 in its native environment. To improve their binding properties by increasing avidity, we have generated bivalent Nbs, a format that has previously significantly improved antigen recognition for other Nbs (Virant et al, 2018; Fagbadebo et al, 2022). Accordingly, we found that bivD7 is able to specifically stain endogenous Drp1 in fixed cells. Most importantly, by site-directed fluorophore conjugation, we generated a probe with a substantially smaller linkage error compared with conventional antibodies, applicable for super-resolution STORM imaging of Drp1. Commonly applied labelling strategies for SRM include direct (labelled primary antibody) and indirect (labelled secondary antibody) immunofluorescence techniques or

genetically encoded fluorescent protein fusions. The linkage error, which describes the distance between the biological sample and the detectable moiety, is determined by the size of the structure used to label the protein of interest (e.g. the antibody) and the random distribution of fluorescence emitters on this structure. This can induce a fluorescence displacement of up to ~30 nm causing mislocalization of the target structure coordinates (Carrington et al, 2019; Fruh et al, 2021). As demonstrated, the bivD7 conjugated to AlexaFluor647 enables for the first time super-resolved imaging of endogenous Drp1 complexes located in the cytoplasm and on mitochondria.

Recently, it was reported that labelling of Drp1 with genetically fused tags including fluorescent proteins such as GFP alters its

oligomerization state and consequently affects its function (Montecinos-Franjola et al, 2020). Therefore, a tag-free approach is desirable to minimize artefacts when studying Drp1 in living cells. In this context, transiently binding Cbs are known as versatile probes to visualize endogenous antigens in living cells with high spatial and temporal resolution (Rothbauer et al, 2006; Burgess et al, 2012; Maier et al, 2015; Panza et al, 2015; Wegner et al, 2017). Here, we reformatted D7 and D63 into intracellularly functional Cbs and monitored the dynamics of endogenous Drp1 by fluorescent time-lapse imaging of living cells. Considering that Drp1 is mainly diffusely localized in the cytoplasm, we had to face the challenge of distinguishing between the signals originating from Drp1-binding Cbs and unbound Cbs, a problem that is constantly arising when using genetically encoded imaging probes expressed from a constitutive promoter (Wagner & Rothbauer, 2020). Several approaches have been developed to reduce the levels of unbound intrabodies and Cbs (Sibler et al, 2005; Gross et al, 2013; Tang et al, 2016; Keller et al, 2018). Here, we introduced an N-terminal arginine residue, which was previously reported to convey a fast proteasomal degradation of unbound Cbs in the cytoplasm via the N-end rule (Varshavsky, 2005; Keller et al, 2018). The improved signal-to-noise ratio of these turnover-accelerated Drp1-Cbs now enables for the first time the visualization of the drug-induced relocalization of endogenous cytoplasmic Drp1 towards mitochondrial fission sites in living cells. In addition to imaging, the Nbs can be further adapted as building blocks of, for example, Drp1 modulating functional intrabodies for extended research applications. As an example, we started to develop Drp1-Nbs as inducible degrons for targeted degradation of Drp1 in living cells. In summary, currently shown as a proof-of-principle study, we are convinced that the herein-presented Nbs open up new opportunities for comprehensive and detailed studies of Drp1 and its pathophysiological role as a key regulator of mitochondrial fission in advanced experimental settings.

Materials and Methods

Nanobody library construction

Alpaca immunization with recombinant Drp1 as well as Nb library generation was performed as described before (Rothbauer et al, 2006). Animal immunization was authorized by the government of Upper Bavaria (Permit number 55.2-1-54-2532.0-80-14). Briefly, an alpaca (*V. pacos*) was immunized with recombinant human Drp1 (Drp1). An initial priming dose of 0.48 mg Drp1 was followed by booster injections of 0.24 mg each in the 3rd, 4th, 7th, and 12th wk. Serum samples of 20 ml were taken in the 9th wk of immunization for analysis of seroconversion by ELISA. 13 wk after the first dose, 100 ml blood was drawn, and the lymphocytes isolated by Ficoll gradient centrifugation using the Lymphocyte Separation Medium (PAA Laboratories GmbH). Total RNA was isolated using TRIzol (Life Technologies) followed by reverse transcription of mRNA into cDNA using a First-Strand cDNA Synthesis Kit (GE Healthcare). To isolate the Nb repertoire, three nested PCR reactions were performed with the following primers: (i) CALL001 and CALL002, (ii) forward primer

set FR1-1, FR1-2, FR1-3, FR1-4 and reverse primer CALL002, and (iii) forward primer FR1-ext1 and FR1-ext2 and reverse primer set FR4-1, FR4-2, FR4-3, FR4-4, FR4-5, and FR4-6 to introduce SfiI and NotI restriction sites. The sequences for all primers (synthesized by Integrated DNA Technologies) used in this study are listed in Table S6. The amplified cDNA comprising the Nb repertoire was then subcloned into the pHEN4 phagemid vector (Arbabi-Ghahroudi et al, 1997) using the SfiI/NotI restriction sites thereby generating the Drp1-Nb library.

Nanobody screening

To select for Drp1-specific Nbs, *E. coli* TG1 cells containing the Drp1-Nb library in pHEN4 were infected with the M13K07 helper phage. Next, the hereby generated Nb-presenting phages were isolated from culture supernatant by PEG precipitation and 1×10^{11} phages were used for subsequent biopanning. In each selection round, extensive blocking was carried out with 5% milk or BSA in PBST (PBS, 0.05% Tween 20, pH 7.4) (Pardon et al, 2014). To deplete non-specific binders, phages were first added to Nunc Immuno MaxiSorp tubes (Thermo Fisher Scientific) coated with 10 µg/ml GFP before transferring them to tubes coated with 10 µg/ml Drp1 or 10 µg/ml GFP as non-related antigen for 2 h at RT. Next, unbound phages were removed by washing with stringency increasing each round. Bound phages were eluted with 100 mM triethylamine (pH 12.0) and immediately neutralized with 1 M Tris-HCl pH 7.4. After each round of panning, log phase *E. coli* TG1 cells were infected with the eluted phages and grown on selection plates to rescue enriched phages. Drp1-specific phage enrichment was monitored by determining the CFUs after each round. After two rounds of panning, 260 individual clones were randomly selected and screened by phage ELISA using immobilized Drp1 or GFP as control. HRP-labelled anti-M13 monoclonal antibody (GE Healthcare) and 1-Step Ultra TMB solution (Thermo Fisher Scientific) were used for detection of bound phages. The reaction was stopped with 100 µl of 1 M H₂SO₄ and the signal was detected on a plate reader (Pherastar) at 450 nm. Phage ELISA-positive clones were defined by a twofold signal above the control.

Expression plasmids

For bacterial Nb expression, Nb encoding cDNA were cloned into the pHEN6C vector as previously described (Rothbauer et al, 2008). For mammalian expression of bivalent Nbs, cDNAs comprising the two coding sequences of the respective Nbs connected by a flexible Gly-Ser linker [(G₄S)₄] and a C-terminal sortase tag (L-P-E-T-G) followed by a His₆-tag were generated and inserted into pCDNA3.4 expression vector variant comprising N-terminal signal peptide for secretion (Wagner et al, 2021) as following: For bivD7, each Nb sequence was amplified separately by PCR using bivD7-1 and bivD7-2 or bivD7-3 and downEcoRI-rev primer pairs. Next, both Nbs were fused by overlap-extension PCR using the primers bivNshort_for and downEcoRI-rev. For bivD63 cloning, the primer pairs bivD63forN and bivNtermGS-rev as well as bivD63forC and downEcoRI-rev were used followed by bivD63_fusion and downEcoRI-rev for overlap-extension PCR. Finally, the resulting sequence was inserted into pCDNA3.4 via restriction digestion with

Esp3I and EcoRI. To generate unmodified Cbs, Nb sequences were cloned into pTagRFP by BglII and HindIII restriction digest as previously described (Panza et al, 2015). For turnover-accelerated Cbs, the Nb sequence was inserted via PstI and BspEI into the previously described pEGFP-Ubi-R-3xGS-tagRFP plasmid (Keller et al, 2018). The coding sequence of Drp1 was amplified from pAcGFP-Drp1 plasmid (Jenner et al, 2022) with Drp1_GTPaseFor and Drp1_C_term_rev and cloned into pEGFP-N1 backbone. For deletion constructs for domain mapping, all sequences were amplified from AcGFP-Drp1 and cloned into pEGFP-N1 with Drp1-GTPaseFor forward primer and the respective reverse primer: Drp1-VD-rev (GTPase-MD-VD), Drp1-MD-rev (GTPase-MD), Drp1-GTPase-rev (GTPase). The correct sequences of all constructs were confirmed by Sanger sequencing. All expression constructs used in this study are listed in Table S7.

Protein expression and purification

Monovalent Drp1-Nbs were expressed and purified as previously described (Rothbauer et al, 2008; Wagner et al, 2021). Bivalent Drp1-Nbs were produced using the ExpiCHO system (Thermo Fisher Scientific) according to the manufacturer's instructions (Wagner et al, 2021; Fagbadebo et al, 2022). Purity of produced Nbs was assessed using SDS-PAGE. Thus, protein samples were denatured (10 min, 95°C) with 2x SDS-sample buffer (60 mM Tris-HCl, pH 6.8; 2% [wt/vol] SDS; 5% [vol/vol] 2-mercaptoethanol; 10% [vol/vol] glycerol, 0.02% bromophenol blue) and gels were stained with InstantBlue Coomassie (Abcam). Protein concentrations were determined using Bradford and spectrophotometer measurements. Drp1 was purified as described in (Jenner et al, 2022). Briefly, DRP1 (isoform 1) was expressed from a pTYB2 vector in *E. coli* BL21-CodonPlus (DE3)-RIPL. Bacteria were grown at 37°C in LB medium containing 100 µg/ml ampicillin and 35 µg/ml chloramphenicol to an OD_{600 nm} of 0.6. Protein expression was induced using 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at 14°C for 18 h. Cell pellets were resuspended in 20 mM HEPES/KOH pH 7.4, 500 mM NaCl and 1 mM MgCl₂ containing 1 mM PMSF, 1 µg/ml DNase I (Roche Diagnostics), and protease inhibitor (Complete; EDTA-free Protease Inhibitor Mixture; Roche Applied Science), homogenized by mechanical rupture and cell debris was removed by centrifugation. The supernatant was purified using chitin resin affinity purification (New England Biolabs, Inc.). Drp1 was eluted by cleavage from the affinity resin using 30 mM DTT at pH 8 for 48 h at 4°C and further purified using anion exchange chromatography (HiTrap Q FF, GE Healthcare) at pH 8. Pure elution fractions were pooled and dialyzed against 20 mM HEPES/KOH pH 7.4, 500 mM NaCl and 1 mM MgCl₂. Purity was assessed using SDS-PAGE as described and protein concentration was determined using Bradford and spectrophotometer measurements. Purified protein was stored with 20% (vol/vol) glycerol.

Affinity measurements by biolayer interferometry

Binding kinetics analysis of Drp1-Nbs was performed using the Octet RED96e system (Sartorius) according to the manufacturer's recommendations. In brief, streptavidin-coated biosensor tips (SA,

Sartorius) were incubated for 40 s with 1.7–10 µg/ml biotinylated Drp1-Nbs diluted in Octet buffer (HEPES, 0.1% BSA). In the association step, a four-step twofold dilution series of Drp1 starting from 125 nM (D7), 62.5 nM (D63) or 120 nM (bivD7 and bivD63) were applied for 300 s followed by dissociation in Octet buffer for 720 s. Each concentration was normalized to a reference applying Octet buffer only for association. Data were analysed with the Octet Data Analysis HT 12.0 software using the 1:1 ligand model and global fitting.

Drp1 GTPase activity measurements

GTPase activities for Drp1 were measured using a continuous, regenerative assay described by (Ingberman & Nunnari, 2005). In brief, Drp1 (100 nM final) was incubated with 0, 10, 100 or 1,000 nM D7, D63, or GFP Nb as negative control. Upon addition of 1 mM DTT, 1 mM PEPK, 600 µM NADH, ≥20 U/ml PK/LDH (all Sigma-Aldrich) and 1 mM GTP, GDP (both Jena Bioscience), or no nucleotide, the reactions were started with the final buffer containing 20 mM Hepes pH 7.4, 150 mM KCl, 2 mM MgCl₂. Upon thermal equilibration of the microtiter plates, NADH absorbances were measured for 120 min using a Safire Tecan-F129013 microplate reader (Tecan) operating at 340 nm and 37°C. Using BSA without nucleotide or GDP for rapid NADH depletion, absorbances were converted into NADH concentrations. GTPase rates were calculated using linear fits between 30 and 120 min of the traces.

Cell culture and transfections

U2OS and HEK293 cell lines were obtained from ATCC (CRL3216, HTB-96); the HeLa Kyoto cell line (Cellosaurus no. CVCL_1922) was acquired from S. Narumiya (Kyoto University, Japan). Drp1 KO HeLa cells were generated in and provided by the laboratory of Thomas Langer (Max Planck Institute, Cologne, Germany). For mycoplasma testing, the mycoplasma kit Venor GeM Classic (Minerva Biolabs) and Taq polymerase (Minerva Biolabs) were applied. No additional authentication was performed as this study does not contain any cell-line-specific experiments. Culturing of cell lines was carried out by standard protocols. In brief, cells were grown in DMEM high glucose, pyruvate, GlutaMax (Thermo Fisher Scientific) with 10% (vol/vol) FCS (Thermo Fisher Scientific) and 1% (vol/vol) penicillin/streptomycin (Thermo Fisher Scientific). For routine passaging of cells, 0.05% trypsin-EDTA (Thermo Fisher Scientific) was used. Cells were cultivated at 37°C and 5% CO₂ in a humidified incubator. U2OS cells were transiently transfected with Lipotectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's recommendations. HEK293 and HeLa cells were transfected with Polyethyleneimine (PEI, Sigma-Aldrich) as previously described (Braun et al, 2016).

Nanobody immobilization on NHS-sepharose matrix

1.2 mg Drp1-Nbs per 1 ml NHS-Sepharose (Cytiva) were immobilized according to the manufacturer's instructions.

Sortase labelling of nanobodies

For sortase coupling, 50 μ M Nb, 250 μ M sortase peptide (H-Gly-Gly-Gly-propyl-azide, synthesized by AG Maurer, University of Tübingen) dissolved in sortase buffer (50 mM Tris, pH 7.5, and 150 mM NaCl) and 10 μ M sortase were mixed in coupling buffer (sortase buffer with 10 mM CaCl₂) and incubated for 4 h at 4°C. Uncoupled Nb and Sortase were removed by immobilized metal affinity chromatography, and the excess peptide was depleted via Amicon Ultra Centrifugal Filters (Merck Millipore). To label the azide-coupled nanobody with a fluorophore, SPACC (strain-promoted azide-alkyne cycloaddition) click chemistry reaction was applied. For this, fivefold molar excess of dibenzocyclooctyne-AlexaFluor647 (DBCO-AF647) (Jena Bioscience) was added to the azide-coupled Nb followed by incubation for 2 h at room temperature and subsequently purified by dialysis and hydrophobic interaction chromatography.

Mammalian cell lysis and protein extraction

For intracellular immunoprecipitation (IC-IP), 2.5–3 $\times 10^6$ HEK293 cells were seeded in a 100 mm culture dish (P100). The next day, cells were transfected with the chromobody-encoding plasmid. After 24 h, cells were harvested at 90% confluency. This workflow was also followed for the domain mapping constructs. For immunoprecipitations (IPs) using nanotraps, T175 flasks or P100 dishes of HEK293, U2OS or HeLa cells were harvested at ~90% confluency. HEK293 cells were detached by light pipetting and U2OS or HeLa cells by trypsinization. Subsequently, the cells were washed with 1 ml PBS, and the pellets were stored at –80°C until lysis. For lysis, T175 flask pellets were resuspended in 600 μ l, P100 pellets in 200 μ l lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8; 0.5% Triton X-100; 1 μ g/ μ l DNaseI; 2.5 mM MgCl₂; 2 mM PMSF; 1x Protease inhibitor Mix M [Serva]). The lysate was passed through 20G, 23G, and 27G needle gauges multiple times with intermediate vortexing and 10 min incubation steps on ice. Subsequently, the lysate was centrifuged for 10 min at 18,000g at 4°C. The supernatant was transferred to a fresh tube and diluted 1.5-fold with dilution buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8; 2 mM PMSF).

Immunoprecipitation

Soluble protein extracts were generated from HEK293, HeLa and U2OS cells as described above. For analysis of the input (IN), 20 μ l of the lysate was mixed with an equal amount of 4x SDS-Sample buffer. For IP, 80 μ l slurry of each nanotrap was equilibrated in IP buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8) and added to equal amounts of lysate. As positive (domain mapping) or negative control (precipitation of endogenous Drp1), 80 μ l GFP-Trap slurry (ChromoTek) was used. For IC-IP, 80 μ l of RFP-Trap slurry (ChromoTek) was used. Soluble protein extracts were incubated with the nanotraps overnight at 4°C on an end-over end rotor. For positive control (precipitation of endogenous Drp1), 4 μ l anti-Drp1 antibody was added to the lysate and after overnight incubation at 4°C 80 μ l equilibrated Protein A/G sepharose slurry were added followed by additional 3–4 h incubation at 4°C. Beads were harvested by centrifugation (2,000g,

2 min, 4°C) and supernatant (non-bound sample) was mixed 1:1 with 4x SDS-sample buffer. The beads were washed three times with 500 μ l wash buffer (IP buffer with 2 mM PMSF, for MS samples in addition to 1x protease inhibitor mix and 0.05% Tween20). With the third wash, beads were transferred into a fresh tube. The beads were boiled in 60 μ l 2x SDS-sample buffer for 10 min at 95°C and the supernatant was transferred into a fresh tube (bound sample).

For immunoblotting of IP samples, 1% of input and non-bound and 33% of the bound sample were loaded on an SDS-Page and blotted on nitrocellulose membrane as previously described (Fagbadebo et al, 2022). Immunoblots were probed with the following antibodies: anti-Drp1, anti-TagRFP, anti-GAPDH (Fig S14A–C). All antibodies used in this study are listed in Table S8. For immunoblot scanning, a Typhoon-Trio laser scanner (GE Healthcare) was used with excitation 633 nm and emission filter 670 nm BP 30 settings.

Chromobody imaging

wt HeLa cells (8,000 cells/well) or Drp1 KO HeLa cells (11,000 cells/well) were seeded in μ clear 96 well plates (Greiner Bio-One). The next day, cells were transfected with the respective plasmids. 24 h post transfection, cells were stained with 400 nM MitoTracker green (Life Technologies) and 4 μ g/ml Hoechst33258 for 30 min. Afterwards, medium was replaced with live-cell visualization medium DMEMgfp-2 (Evrogen) supplemented with 10% FBS, 2 mM L-glutamine with or without 20 μ M CCCP. Images were taken 10, 70, and 130 min after compound addition with ImageExpress Micro Confocal High Content screening system (Molecular devices) at 40x magnification. During imaging, cells were kept at 37°C and 5% CO₂. Raw microscopy images were adjusted in brightness and contrast using MetaExpress software (64 bit, 6.2.3.733 or higher, Molecular devices).

Immunofluorescence in fixed cells

Cells were seeded in μ clear 96 well plates (Greiner Bio-One) in the same densities as described for Cb imaging. The next day, cells were transfected with GFP-Drp1 plasmid or left untransfected. 24 h post-transfection, cells were washed twice with PBS and subsequently fixed with 3.7% PFA for 10 min at RT. After three times washing with PBS, cells were incubated with TBST for 10 min at RT. After additional washes, cells were blocked with 5% BSA in TBST for 1 h. Afterwards, the cells were incubated with 100–1,000 nM Nb and anti-Drp1 antibody (1:100–1:300) in 5% BSA/TBST overnight at 4°C. Then, unbound Nb and antibody were washed away and the cells incubated with secondary antibody (anti-rabbit labelled with AF647; 1:1,000) and Cy5-conjugated goat anti-alpaca antibody (1:500) for 1 h at RT in 2.5% BSA/TBST. Nuclei staining with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was performed concurrently. After additional washing with TBST and PBS, images were acquired using an ImageExpress Micro Confocal High Content screening system (Molecular devices) at 40x magnification. Raw microscopy images were adjusted in brightness and contrast using MetaExpress software (64 bit, 6.2.3.733 or higher, Molecular devices).

Liquid chromatography-MS sample preparation and measurement

Immunoprecipitation of Drp1 using the D7 and D63 nanotraps were compared with the GFP-Trap (negative control) in three technical replicates. Immunoprecipitation was performed as described above and proteins were subsequently separated by SDS-PAGE (4–12% NuPAGE tris gel [Invitrogen]) for 7 min at 200 V and stained with Coomassie blue. The protein gel pieces were subjected to tryptic digestion as described previously (Shevchenko et al, 2006). Purified peptide samples were measured on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) online-coupled to an Easy-nLC 1200 UHPLC (Thermo Fisher Scientific). Peptides were separated using a 20-cm-long, 75- μ m-inner diameter analytical HPLC column (ID PicoTip fused silica emitter; New Objective) packed in-house with ReproSil-Pur C18-AQ 1.9- μ m silica beads (Dr Maisch GmbH) and eluted using a 60 min segmented gradient from 10–90% of solvents A (0.1% formic acid) and B (80% acetonitrile in 0.1% formic acid) at a constant flow rate of 200 nL/min. The column temperature was maintained at 40°C using an integrated column oven. Peptides were ionized by nanospray ionization and a capillary temperature of 275°C. The mass spectrometer was operated in the positive ion mode. Full MS scans were acquired in a range of 300–1,750 m/z at resolution of 60,000. Seven most intense multiple-charged ions were selected for HCD fragmentation with a dynamic exclusion period of 30 s and tandem MS (MS/MS) spectra were acquired at resolution of 15,000.

Mass spectrometry data analysis

The acquired raw files were processed by MaxQuant software (version 2.0.3.0.) (Cox & Mann, 2008) and searched against the Uniprot *Homo sapiens* database (105,079 entries, downloaded 2022/08/01), *V. pacos* derived nanotraps and commonly observed protein contaminants. For MS and MS/MS, the peptide mass tolerance was set at 4.5 ppm and 20 ppm, respectively. Only two missed cleavages were allowed for the tryptic digestion. Carbamidomethylation (C) was used as fixed modification, whereas oxidation (M) and acetylation (protein N-term) were defined as variable modifications. False discovery rate was set to 1% at both peptide and protein levels. For label-free quantification, a minimum ratio count of two was requested. Intensity-based absolute quantification (iBAQ) was enabled. Downstream analysis of the “proteinGroups.txt” output table was performed in Perseus (version 1.6.15.0). Contaminants, reversed proteins and proteins only identified by site were filtered out. Only proteins that were quantified in two of three replicates of either the bait or control pulldown were retained in the dataset. Missing values were imputed (width 0.3, downshift 1.8). To determine significantly enriched proteins, a multi-volcano analysis (Hawaiian plot) was performed. The s_0 and FDR parameters of the multi-volcano analysis were: for class A (higher confidence, $s_0 = 0.1$, FDR = 0.01) and class B (lower confidence, $s_0 = 0.1$, FDR = 0.05). Additional graphical visualization was performed in the R environment (version 4.1.1) and in GraphPad (version 8.0.1.).

Cell cultivation, seeding, and staining for super resolution microscopy

U2OS wt or U2OS mEGFP-Drp1 cells were cultivated in DMEM (low glucose) supplemented 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin (Invitrogen) at 37°C and 5% (vol/vol) CO₂. For microscopy, the cells were seeded in 35 mm μ -Dish 1.5H glass bottom imaging chambers (for confocal imaging) or on 35 mm 1.5H glass coverslips in a six-well plate (for SMLM) at a density of 3×10^5 cells per dish/well and grown for 18 h. If required, mitochondria were stained using 200 nM MitoTracker Green FM (Invitrogen) for 20 min. Cells were fixed in 4% (vol/vol) PFA in DMEM (pre-warmed to 37°C) for 10 min at RT and washed twice with PBS. To quench unreacted fixative, the cells were incubated in 50 mM NH₄Cl for 15 min followed by permeabilization in 0.25% (vol/vol) Triton X-100 for 8 min. The cells were washed with PBS three times for 5 min and incubated with 1% (wt/vol) BSA in PBS for 1 h to block unspecific binding sites. DRP1 was labelled using 250 nM bivD7 coupled to AlexaFluor 647 (Invitrogen) in 1% (wt/vol) BSA in PBS over night at 4°C protected from light. The cells were washed three times with PBS and stored at 4°C in the dark until imaging.

Confocal fluorescence microscopy

U2OS mEGFP-Drp1 cells were prepared as described above. Confocal fluorescence microscopy images were acquired on an inverted Infinity Line confocal dual-color STED 775 QUAD laser scanning microscope (Abberior Instruments) equipped with a UPLXAP0 60x/1.42 Oil objective (Olympus). mEGFP was excited at 488 nm and AF647 was excited at 640 nm wavelength. Images were acquired with a pixel size of 65 nm and a pixel dwell-time of 5 μ s. The fluorescence emission signal was collected on avalanche photodiode detectors. Raw microscopy images were adjusted in brightness and contrast using Fiji/ImageJ (Schindelin et al, 2012). Colocalization of endogenous mEGFP-Drp1 and bivD7_{AF647} was assessed by calculating the Pearson’s correlation coefficient of the respective emission signals using Just Another Co-localization Plugin (JACoP) (Bolte & Cordelières, 2006) in Fiji/ImageJ (Schindelin et al, 2012). For this, the images were cropped to remove unspecific bivD7 signal outside of the cell and in the nucleus. The experiments are representative for $n = 3$ individual replicates with $n = 11$ cells in total.

Single-molecule localization microscopy

U2OS WT cells were seeded and stained as described above and mounted on custom-made imaging chambers or single concave depression microscopy slides covered with 100–300 μ l imaging buffer (50 mM Tris-HCl pH 8, 10 mM NaCl, 10% [wt/vol] glucose, 35 mM cysteamine [MEA], 0.5 mg/ml glucose oxidase [Sigma-Aldrich] and 40 μ g/ml catalase [Sigma-Aldrich]) and sealed using Twinsil speed dental glue (Picodent). The samples were imaged on a home-built wide-field/TIRF microscope or on a SR GSD (3D) inverted DMI6000 B super-resolution wide-field/TIRF microscope (Leica Microsystems) equipped with a 100x, 1.47 N.A. GSD Objective, 405, 488, 532, and 642 nm excitation lasers and an Andor iXon Ultra 897 EMCCD camera. Image series were acquired with an exposure

time between 30 and 50 ms and an EM gain of 300. AF647 was excited using 640 nm wavelength and the 405 nm activation laser intensity was manually adjusted to keep a constant number of localizations per frame. Typically, 70,000–100,000 frames were recorded. Analysis was performed using the LAS X Software (Leica) and ThunderStorm (Ovesny et al, 2014). Images were rendered using a Gaussian with a width according to the localization precision. Image analysis was performed using Fiji/ImageJ (Schindelin et al, 2012). Images are representative for $n = 6$ individual experiments.

Image analysis and statistics

Image analysis was performed with MetaExpress software (64 bit, 6.23.733 or higher, Molecular devices) for $n > 130$ cells per construct. Using the Custom editor (version 25.13.3 or higher) or the MetaExpress software, we developed an algorithm to determine the average signal intensity per transfected cell. Based on the assumption that cells usually are transfected with both constructs, the GFP-NLS signal was used as a marker for transfection and cell area. Cell average intensity of the TagRFP signal was determined for all GFP-positive cells. Using the DAPI signal in GFP-NLS negative cells, the TagRFP signal intensity was measured for background determination. The average background signal was determined ($n = 2125$ cells) and subtracted from all measurements. A workflow is shown in Fig S15A and B.

Mean and SD were calculated for each condition. For comparison of unmodified and turnover-accelerated Drp1-Cbs, significance was calculated using Kruskal Wallis test with Dunn's multiple comparisons test. For comparison of turnover-accelerated Cbs in wt HeLa and Drp1 KO HeLa cells, significance was calculated using two-way ANOVA with Sidak's multiple comparisons test. Tests were performed using GraphPad Prism software (Version 8 or higher). All graphs were prepared using GraphPad Prism.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al, 2022) partner repository with the dataset identifier PXD051737.

Supplementary Information

Supplementary Information is available at <https://doi.org/10.26508/lsa.202402608>.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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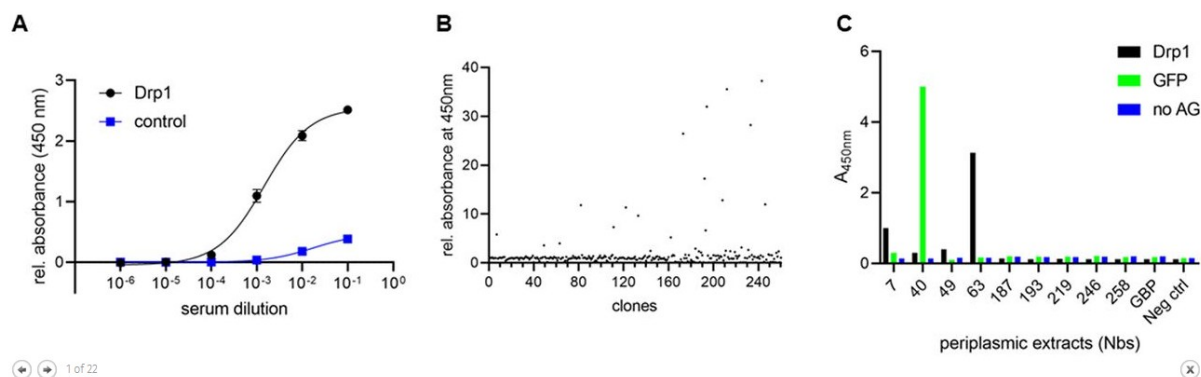
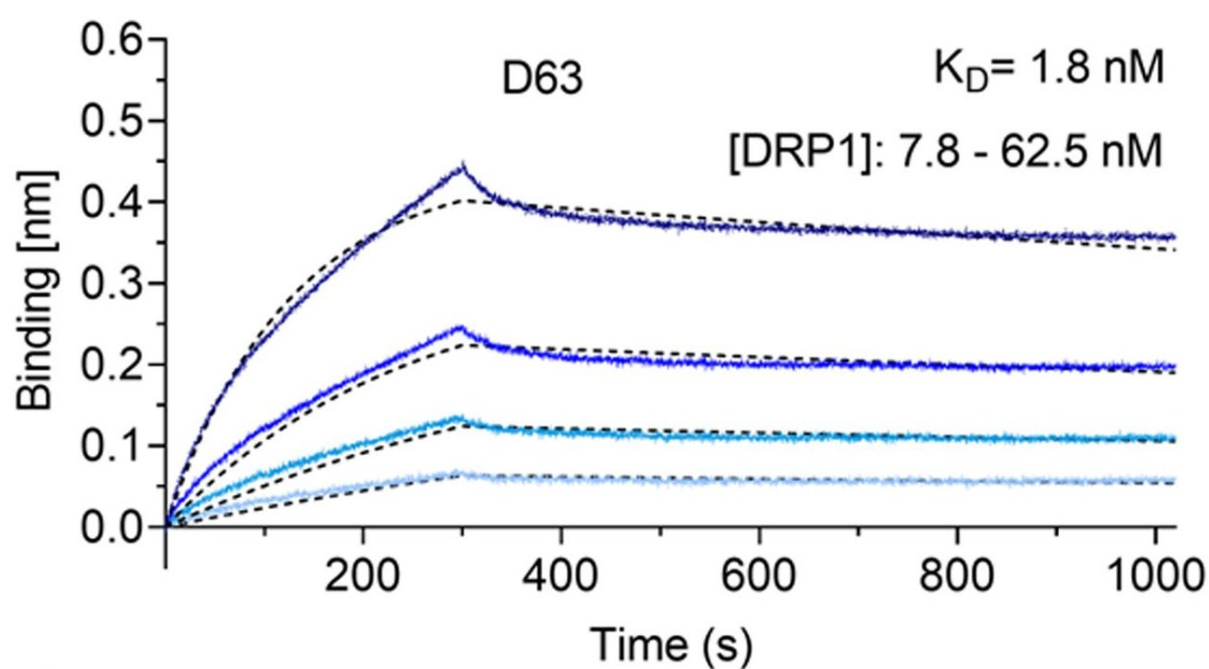


Figure S1.

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Screening for Drp1-specific nanobodies.

(A) Analysis of seroconversion upon vaccination with Drp1. A serum sample of the vaccinated alpaca (*Vicugna pacos*) was collected 13 wk after the first dose. To test for Drp1-specific antibodies, a serum enzyme-linked immunosorbent assay was performed with the indicated dilutions in plates coated either with Drp1 (black) or a non-related His-tagged protein (Sortase_{His6}) (blue) as negative control. Binding of Drp1-specific antibodies was detected by using an anti-heavy chain antibody conjugated to horseradish peroxidase. **(B)** Result of phage ELISA of 260 selected clones to test for binding of Drp1. Signal intensities were normalized to GFP used as negative control. **(C)** Protein ELISA with periplasmic extracts from the eight nanobodies identified by phage ELISA.



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x

Figure S2.

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Affinity measurement of D63.

Bi-layer interferometry-based affinity measurements for D63. Biotinylated D63 was immobilized on streptavidin sensors. Kinetic measurements were performed using four concentrations of purified Drp1 ranging from 7.8 to 62.5 nM (displayed with gradually darker shades of color). The binding affinity (K_D) was calculated from global 1:1 fits shown as dashed lines.

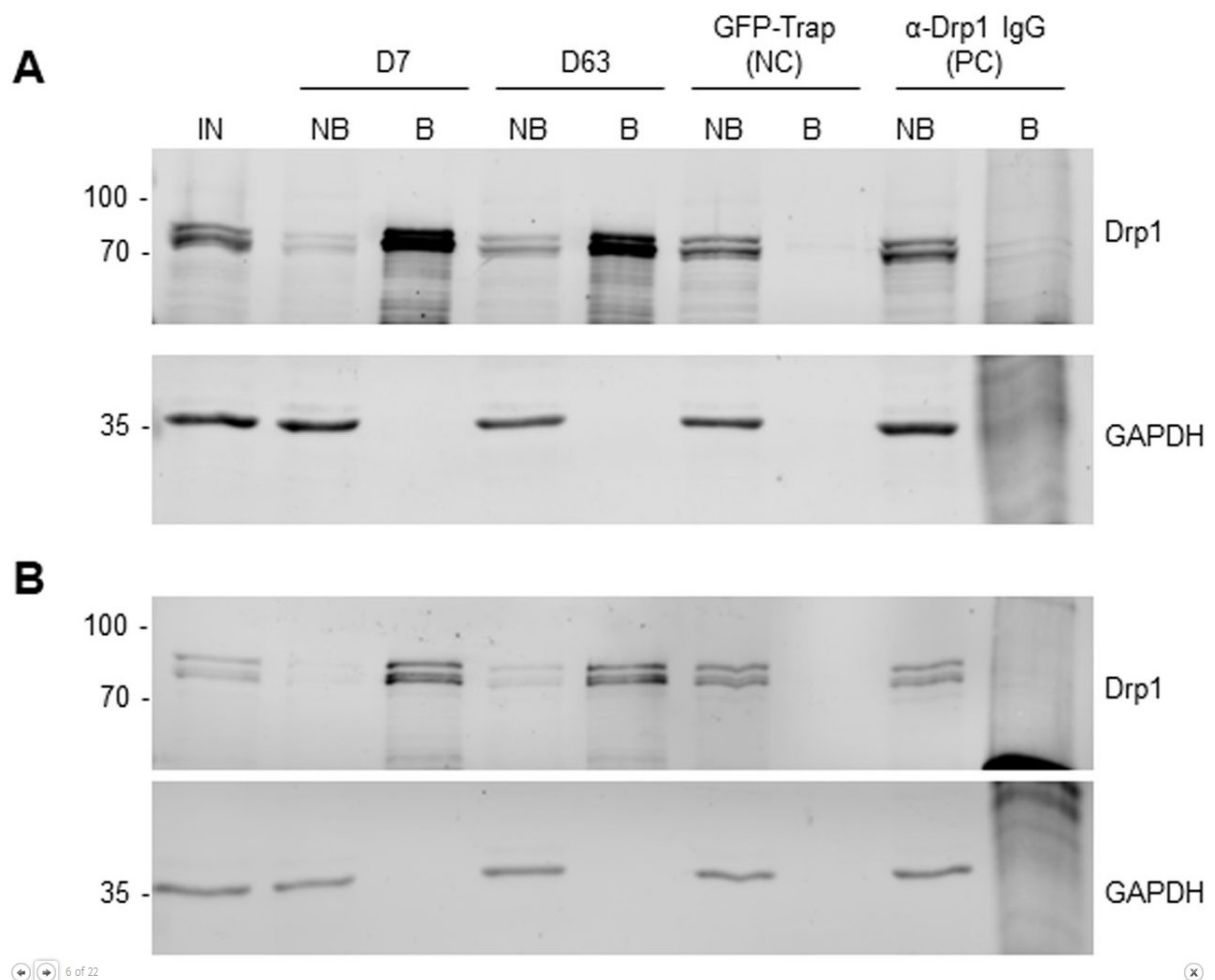


Figure S3.

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Immunoprecipitation of Drp1 from HeLa and U2OS cells.

(A, B) For immunoprecipitation with nanotraps, the soluble protein fraction of HeLa (A) or U2OS cells (B) was incubated with equal amounts of the Drp1 nanotraps (D7, D63). As negative control (NC), the GFP-Trap as a non-specific nanotrap was used. As positive control, anti-Drp1 IgG immobilized on protein A/G sepharose beads was used (PC). Input (IN, 1% of total), non-bound (NB, 1% of total) and bound (B, 33% of total) fractions were subjected to SDS-PAGE followed by immunoblot analysis using antibodies specific for Drp1 (upper panel) and GAPDH (lower panel). Molecular weights in kD are indicated on the left.

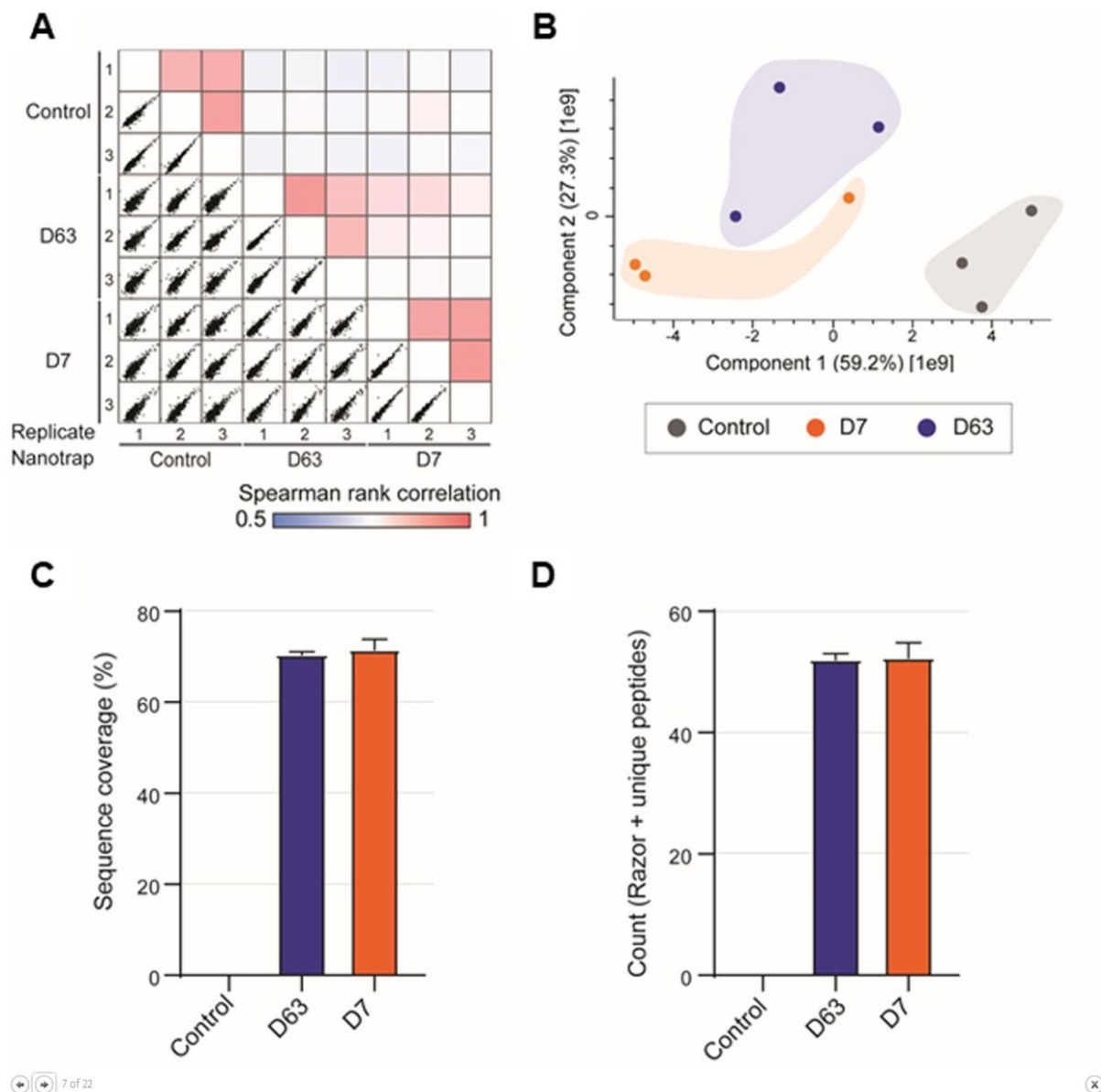


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Enrichment efficiency of Drp1 nanotraps shown for immunoprecipitations from soluble protein fractions of HEK293 cells.

(A) Multi-Spearman correlation between replicates and nanotraps as indicated. **(B)** Principal component analysis reflects the highest similarity between replicates. **(C)** Averaged sequence coverage of Drp1 after precipitation with indicated nanotraps. **(D)** Averaged identification of Drp1 razor and unique peptides corresponding to isoforms 1, 5, 6 or 7. **(C, D)** Shown are the results of three technical replicates ($n = 3$) $\pm$ S.D.

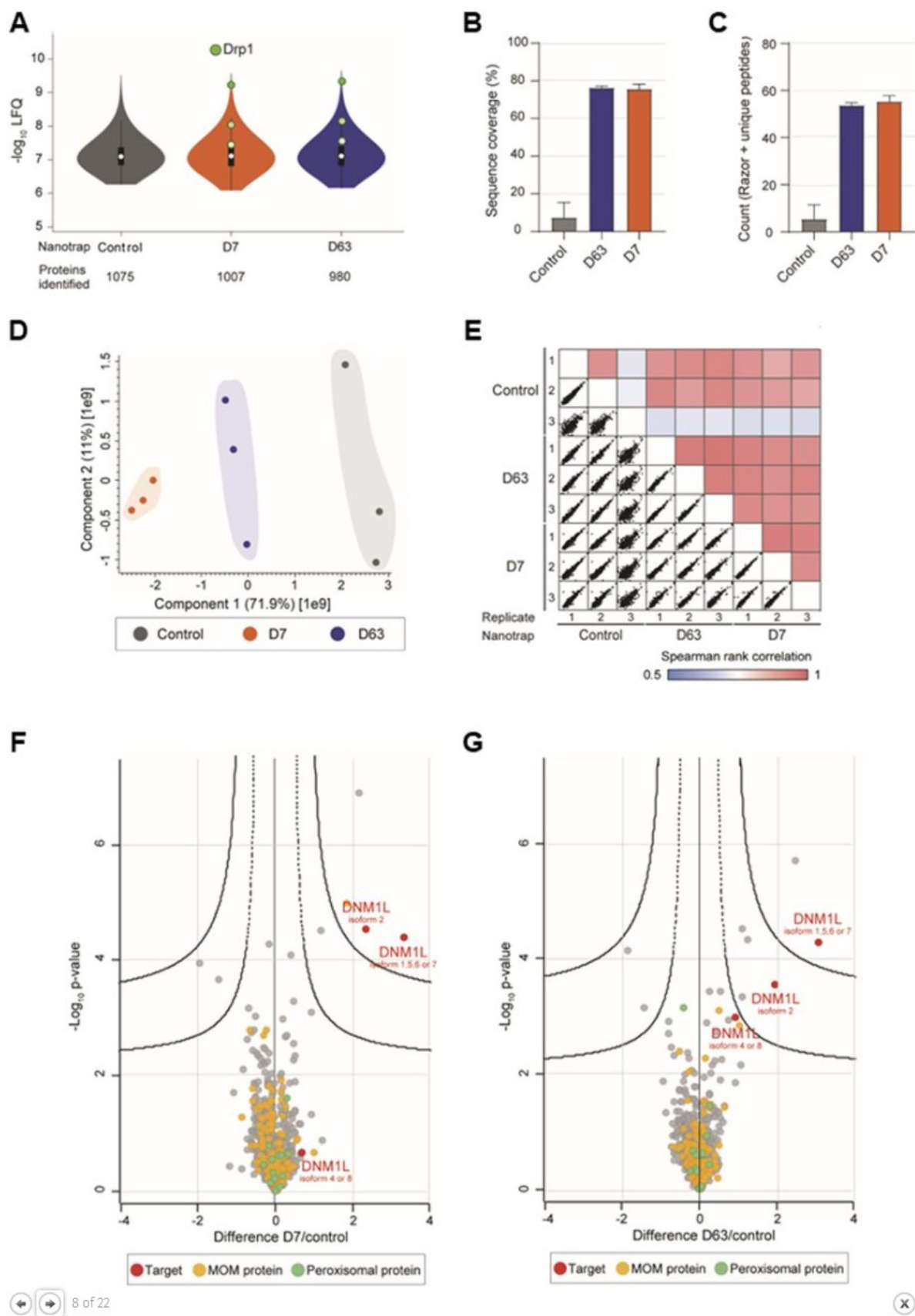


Figure S5.

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Mass spectrometry analysis of the interactome of the Drp1 nanotraps from HeLa cells.

(A) Violin plot of the averaged label free quantification intensities of the proteins identified from immunoprecipitation with the Drp1 nanotraps using HeLa cells. White circles show the median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; polygons represent density estimates of data and extend to extreme values. Drp1 is marked in green.

(B) Averaged sequence coverage of Drp1 after precipitation with indicated nanotraps. **(C)**

Averaged identification of Drp1 razor and unique peptides. **(B, C)** Shown are the results of three technical replicates ($n = 3$) $\pm$ S.D. **(D)** Principal component analysis reflects the highest similarity between replicates. **(E)** Multi-Spearman correlation between replicates and

nanotraps as indicated. **(F, G)** Multi-volcano analysis (Hawaiian plot) of the pull-down of D7 (F)

and D63 (G) against the negative control. Log₂ transformed ratios of the difference between

the Drp1 nanotrap and the control (x axis) are plotted against the log10 transformed *P*-value

(y axis). Drp1 is marked in red, mitochondrial outer membrane proteins are marked in yellow

and peroxisomal proteins in green. Significant interactors can be class A hits (higher

confidence, $s_0 = 0.1$, FDR = 0.01) or class B hits (lower confidence, $s_0 = 0.1$, FDR = 0.05),

thresholds are displayed as a solid line and a dashed line, respectively.

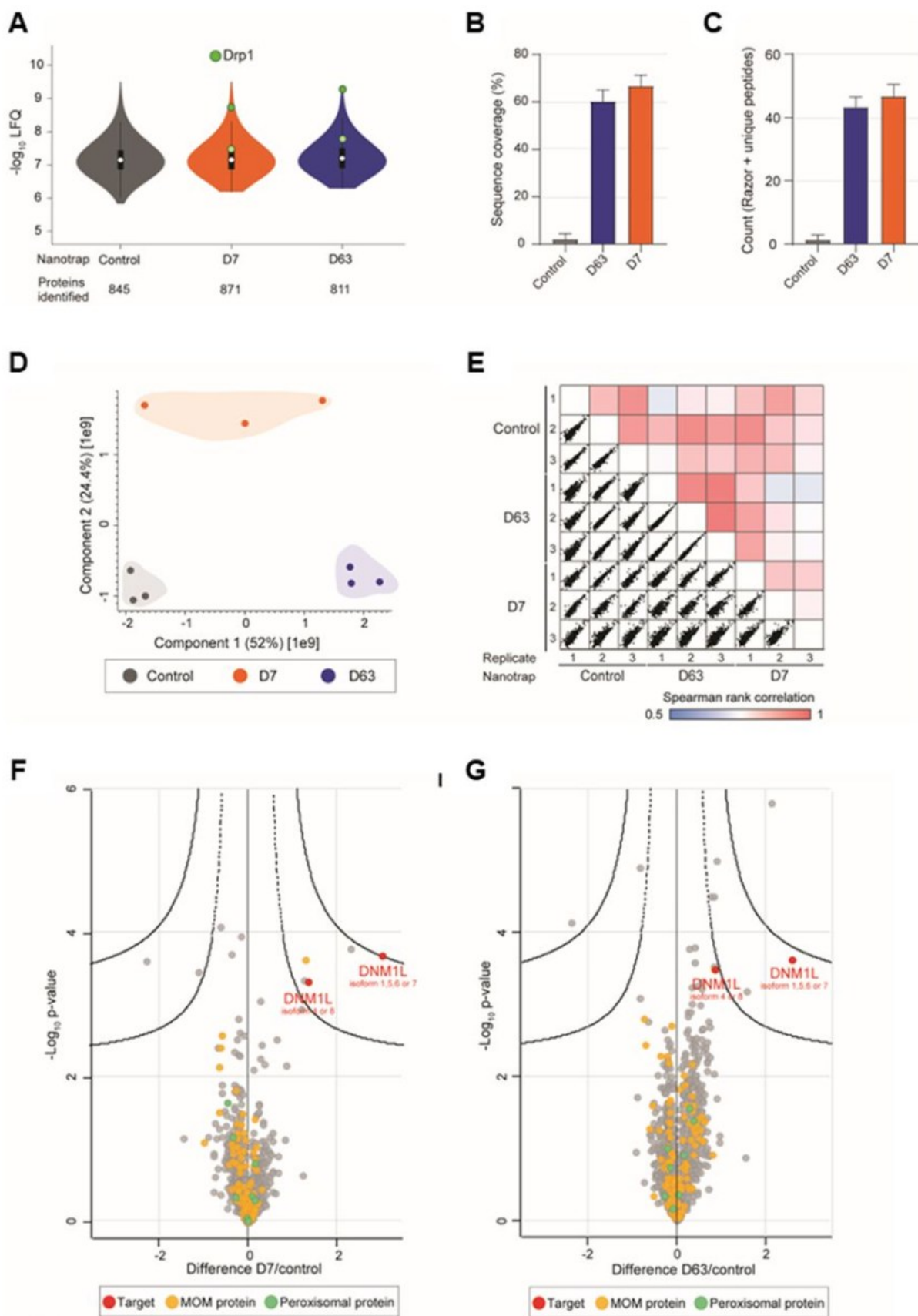


Figure S6.

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Mass spectrometry analysis of the interactome of the Drp1 nanotraps from U2OS cells.

(A) Violin plot of the averaged label free quantification intensities of the proteins identified from immunoprecipitation using U2OS cells. White circles show the median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; polygons represent density estimates of data and extend to extreme values. Drp1 is marked in green. **(B)** Averaged sequence coverage of Drp1 after precipitation with indicated nanotraps. **(C)** Averaged identification of Drp1 razor and unique peptides. **(B, C)** Shown are the results of three technical replicates ($n = 3$) $\pm$ S.D. **(D)** Principal component analysis reflects the highest similarity between replicates. **(E)** Multi Spearman correlation between replicates and nanotraps as indicated. **(F, G)** Multi-volcano analysis (Hawaiian plot) of the pull down of D7 (F) and D63 (G) against the negative control. $\log_2$ transformed ratios of the difference between the Drp1 nanotrap and the control (x axis) are plotted against the $\log_{10}$ transformed P -value (y axis). Drp1 is marked in red, mitochondrial outer membrane proteins are marked in yellow and peroxisomal proteins in green. Significant interactors can be class A hits (higher confidence, $s_0 = 0.1$, FDR = 0.01) or class B hits (lower confidence, $s_0 = 0.1$, FDR = 0.05), thresholds are displayed as a solid line and a dashed line, respectively.

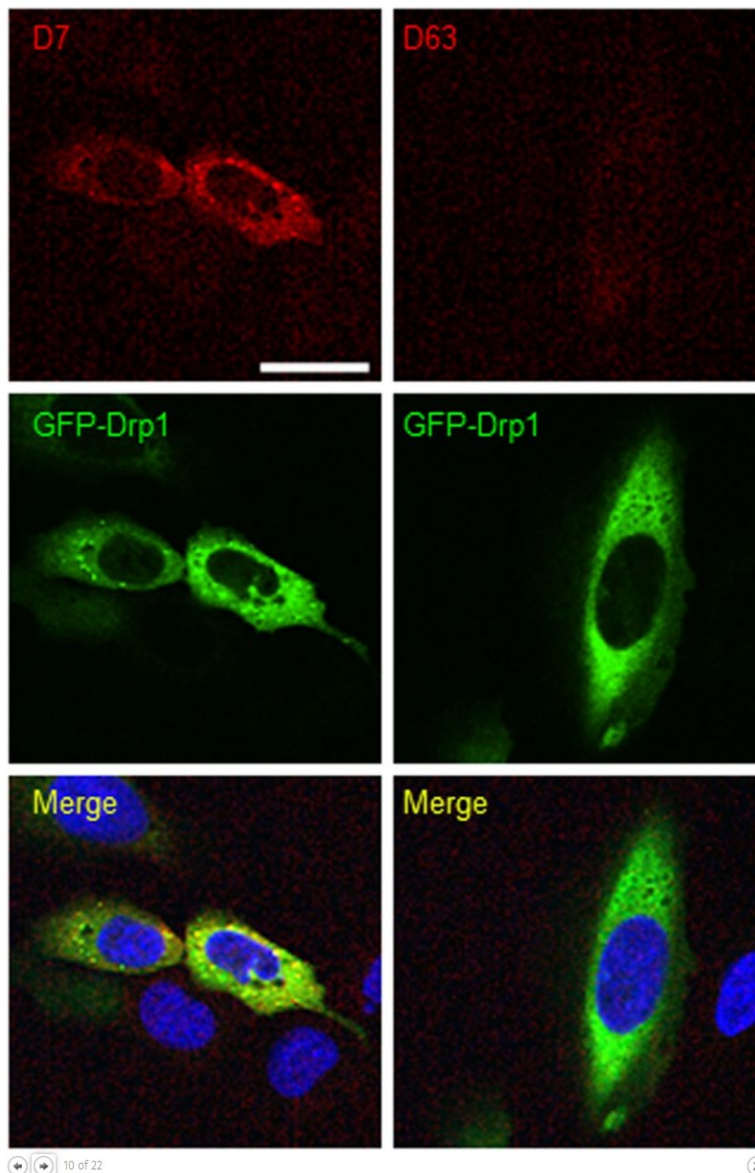


Figure S7.

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Immunofluorescence staining of GFP-Drp1-expressing cells with Drp1-Nbs.

Immunofluorescence detection of Drp1 in fixed and permeabilized U2OS cells transiently overexpressing GFP-Drp1 (green) after staining with D7 and D63 (red) as primary labelling probes. Representative confocal laser scanning microscopy images are shown. Scale bar 25 μ m.

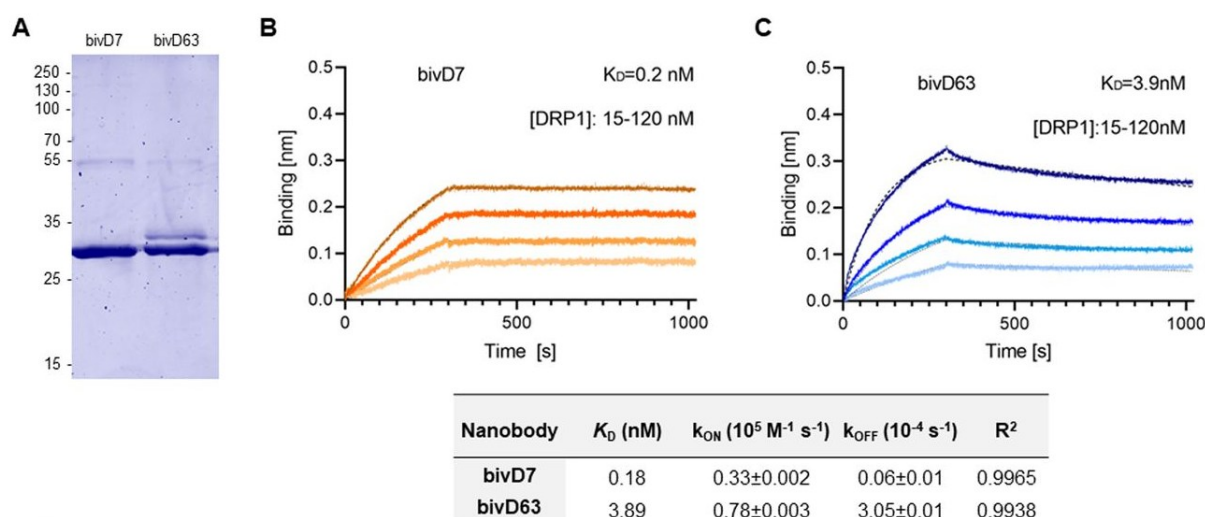


Figure S8.

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Biochemical characterization of bivalent D7 and D63.

(A) Recombinant expression and purification of Drp1 nanobodies (Drp1-Nbs) bivD7 and bivD63 from ExpiCHO cells by immobilized metal affinity chromatography. Coomassie staining of purified Nbs (2 μ g) is shown. Numbers indicate molecular weight (kD). **(B, C)** Biolayer interferometry-based affinity measurements for bivD7 (B) and bivD63 (C). Biotinylated Nbs were immobilized on streptavidin sensors. Kinetic measurements were performed using four concentrations of purified Drp1 ranging from 15–120 nM (displayed with gradually darker shades of color). The binding affinity (K_D) was calculated from global 1:1 fits shown as dashed lines. The table summarizes the affinity analysis of bivD7 and bivD63. Affinities (K_D), association constants (k_{on}) and dissociation constants (k_{off}) are shown as mean $\pm$ SD.

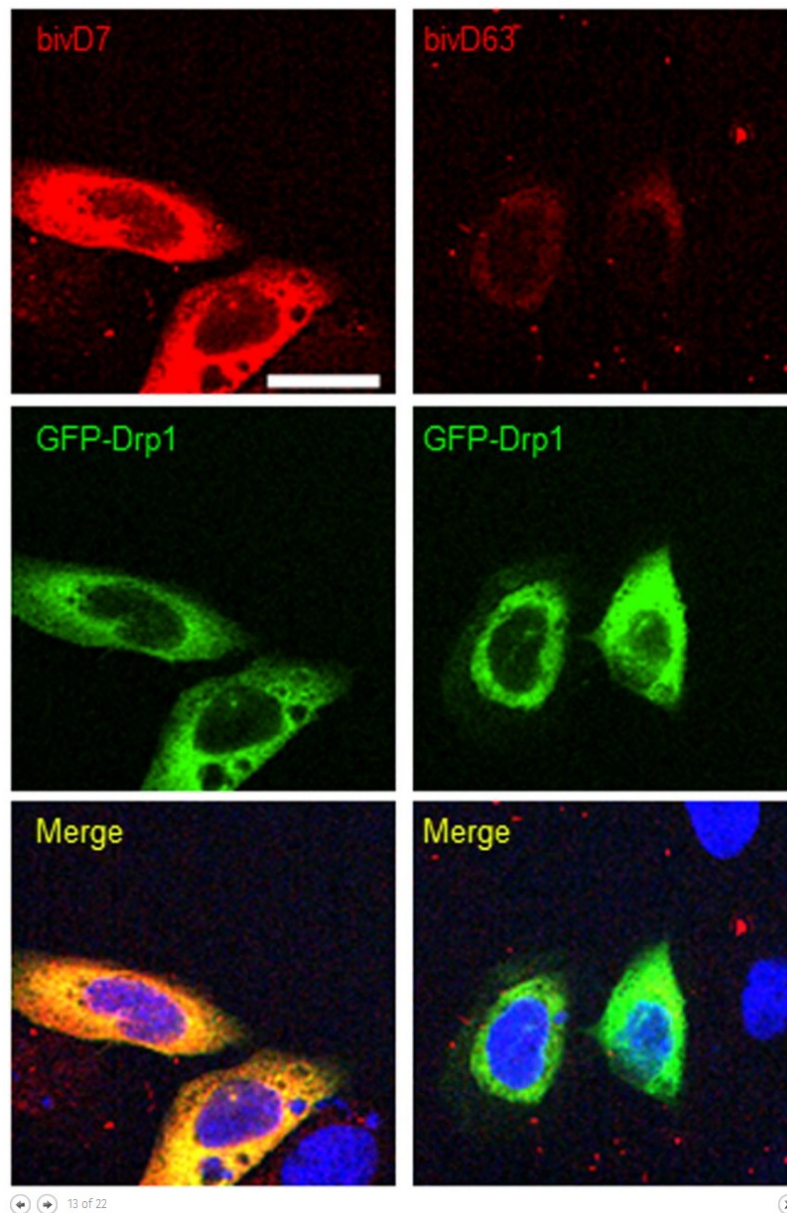


Figure S9.

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Immunofluorescence staining of GFP-Drp1 expressing cells with bivalent Drp1-Nbs.

Immunofluorescence detection of Drp1 after staining with bivD7 and bivD63 (red) as primary labelling probes in fixed and permeabilized U2OS cells transiently overexpressing GFP-Drp1 (green). Representative confocal laser scanning microscopy images are shown. Scale bar 25 μ m.

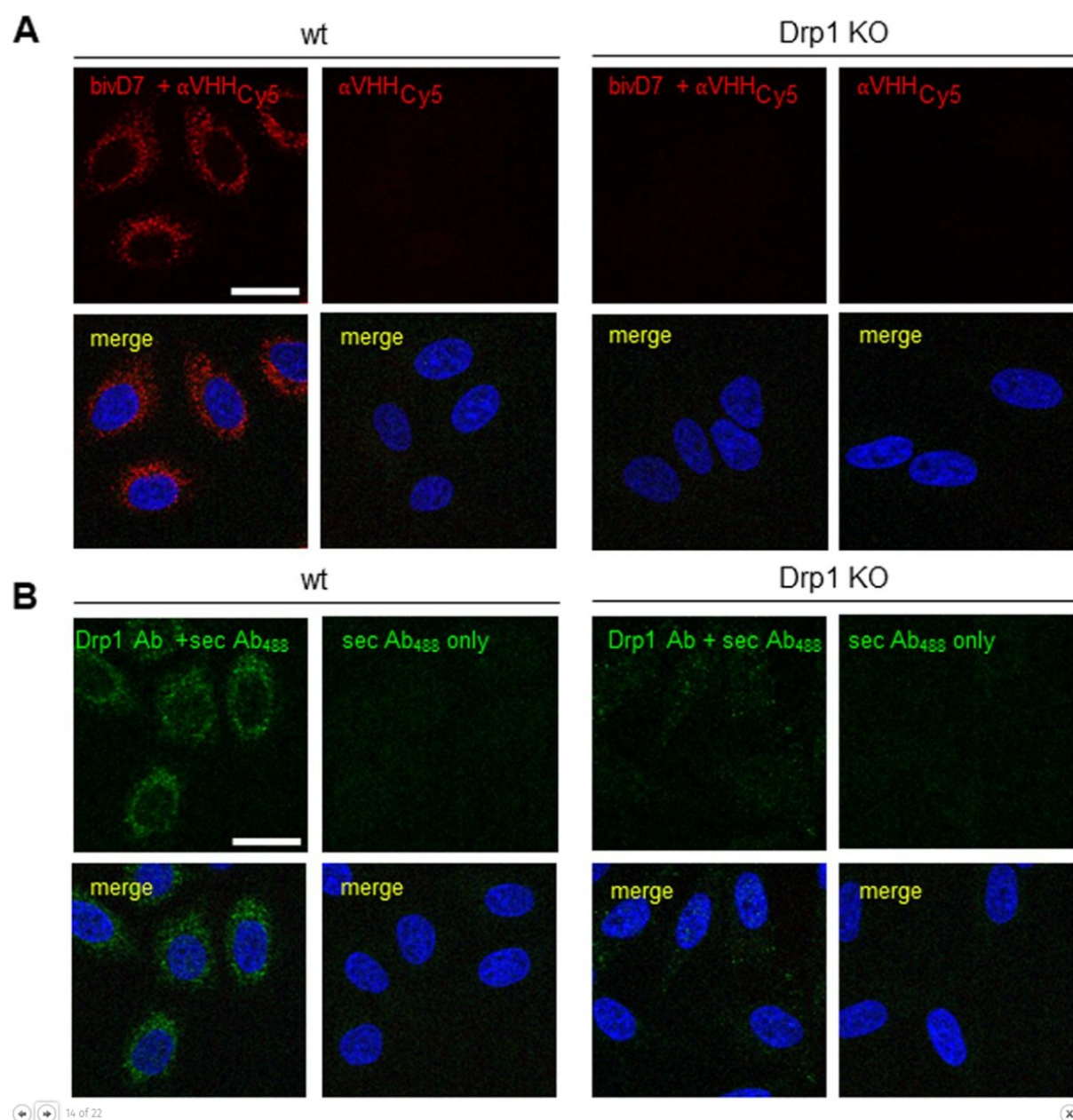


Figure S10.

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bivD7 specifically recognizes endogenous Drp1.

(A, B) Immunofluorescence detection of Drp1 in fixed and permeabilised wt (left panel) or Drp1 KO HeLa cells (right panel) with bivD7 (red) (A) or an anti-Drp1 antibody (Drp1 Ab, green) (B) as primary labelling probes. For control, cells were stained with the respective fluorescently labelled secondary antibodies (sec Ab₄₈₈ or $\alpha\text{VHH}_{\text{Cy5}}$, red) only. Nuclei were stained with DAPI (blue). Representative confocal laser scanning microscopy images are shown. Scale bar 25 μm .

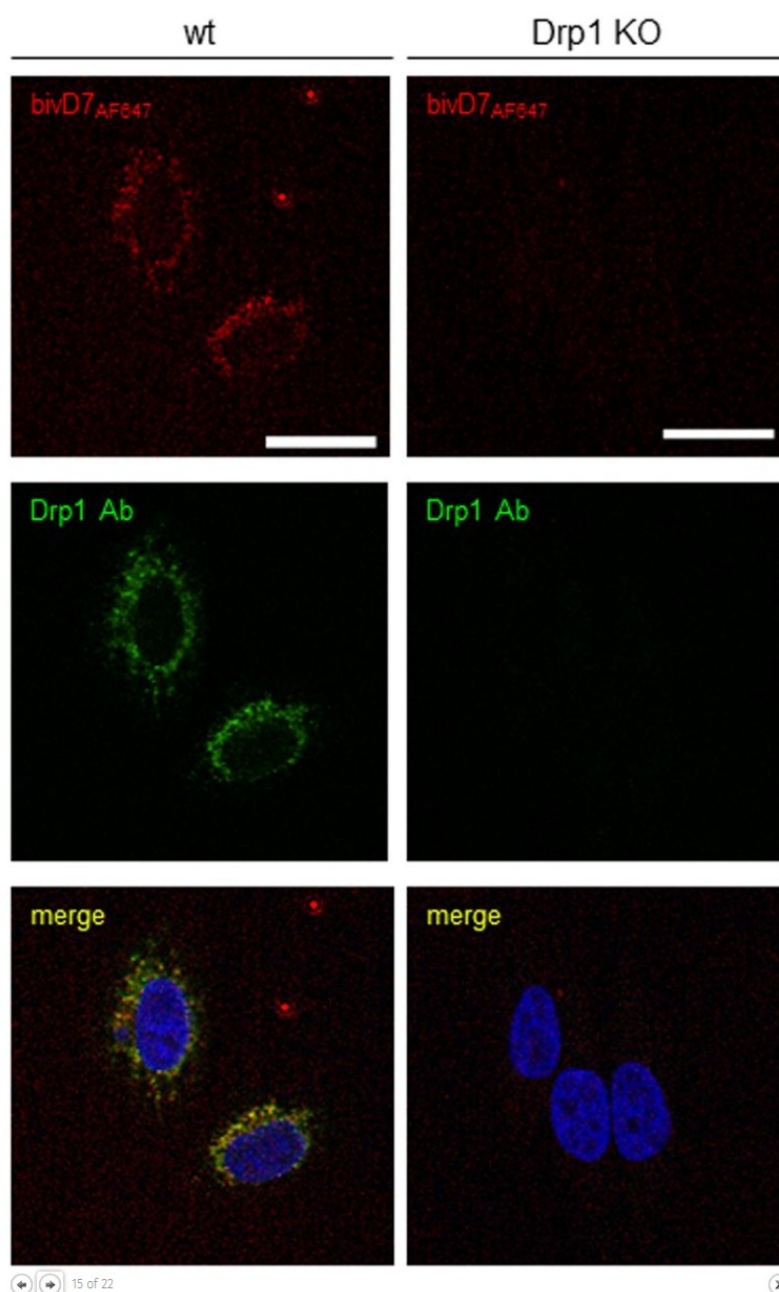


Figure S11.

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Directly labelled bivD7_{AF647} stained endogenous Drp1.

Wt or Drp1 KO HeLa cells were stained with fluorescently labelled bivD7_{AF647} (red). For control, an anti-Drp1 antibody (Drp1 Ab) in combination with an AlexaFluor488-labelled secondary antibody (green) was used. Nuclei were stained with DAPI (blue). Representative confocal laser scanning microscopy images are shown. Scale bar 25 μ m.

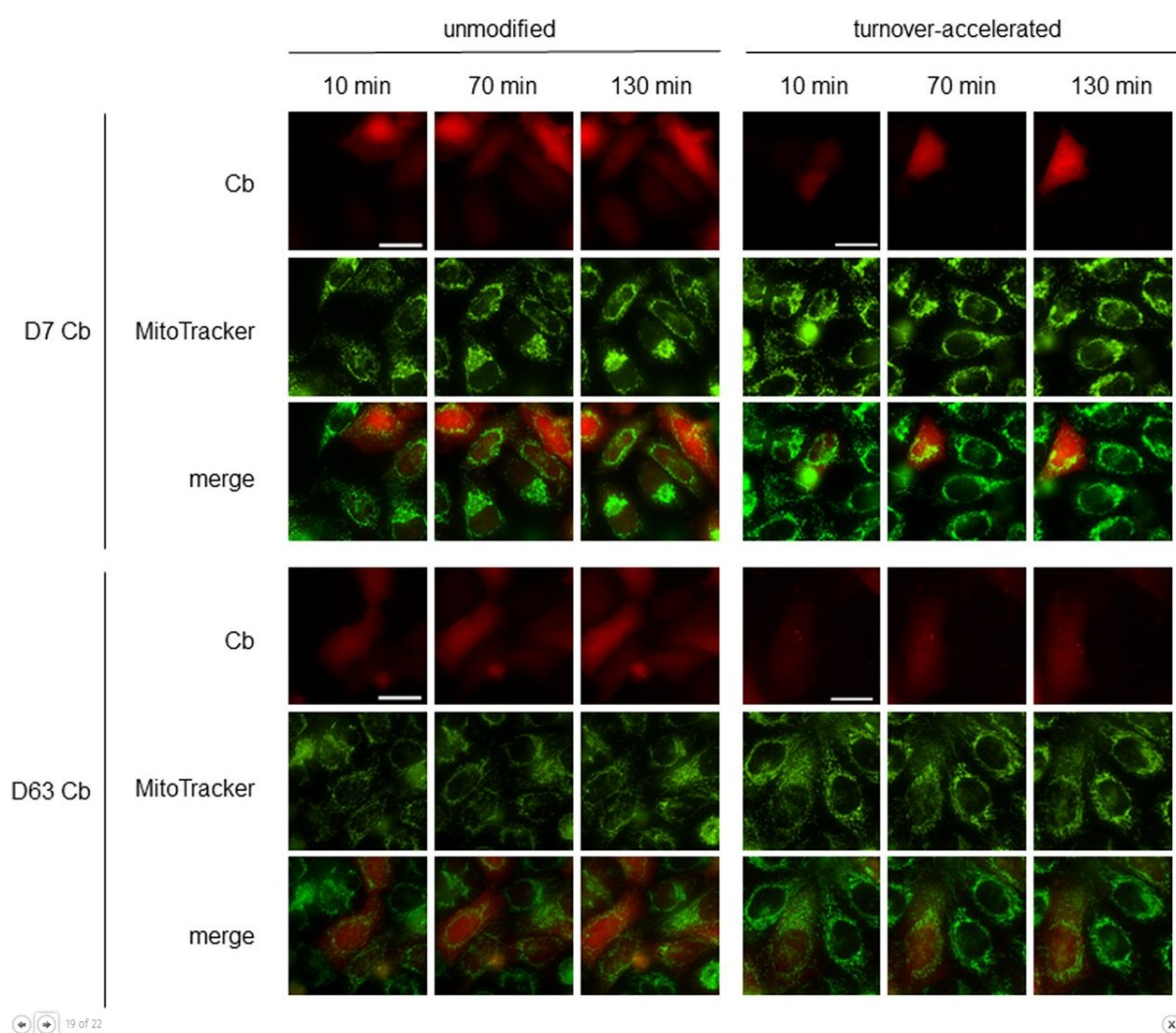


Figure S12.

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Live cell imaging of Drp1-Cbs in wt HeLa cells in the absence of cyanide m-chlorophenylhydrazine.

Time lapse microscopy of wt HeLa cells transiently expressing unmodified or turnover-accelerated Drp1 chromobodies. 1 d post transfection, cells were stained with Hoechst33258 (blue) and MitoTracker green (green). Representative confocal images were taken after 10, 70, and 130 min. Signal intensity was adjusted for each construct. Scale bar 25 μ m.

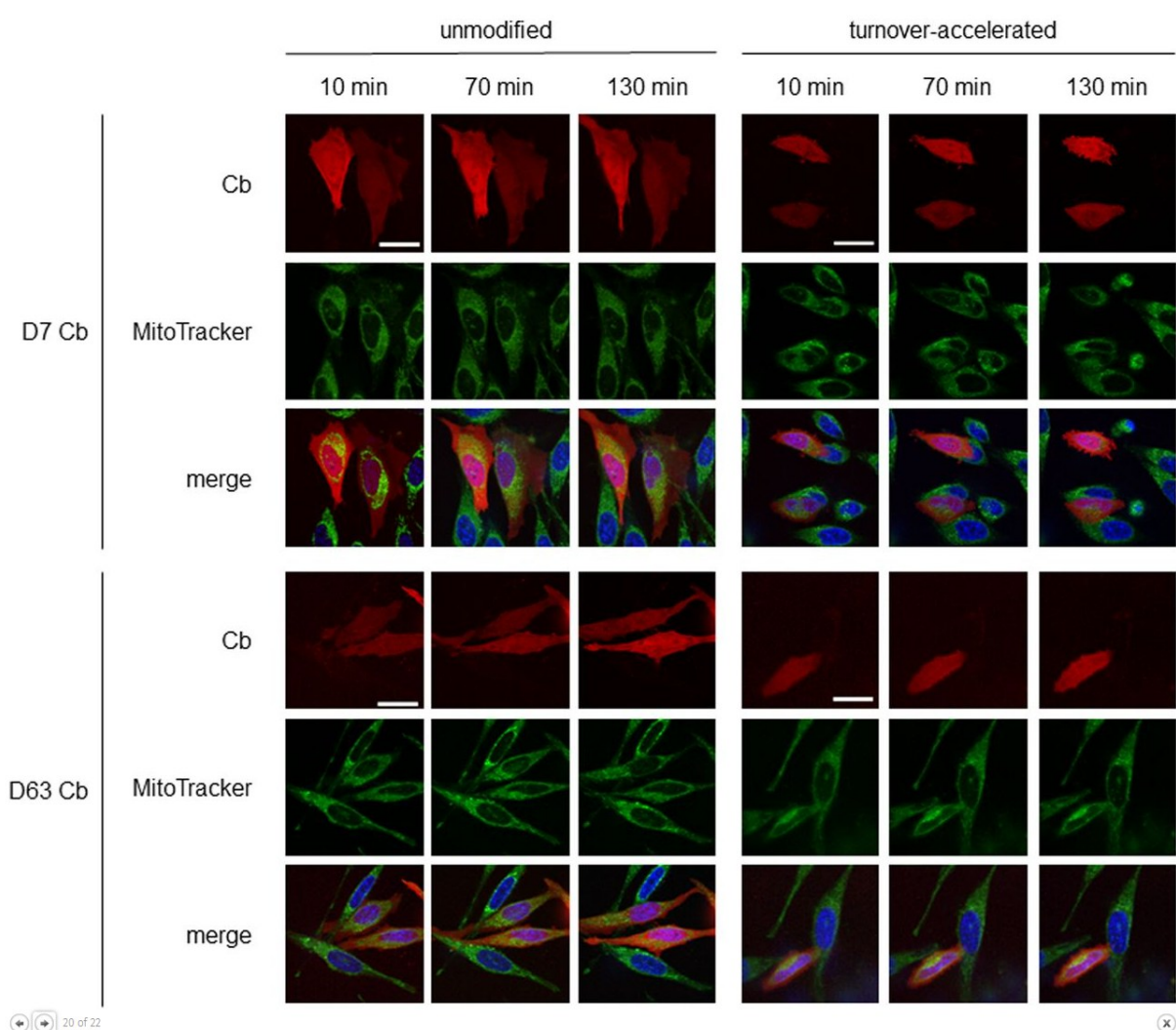


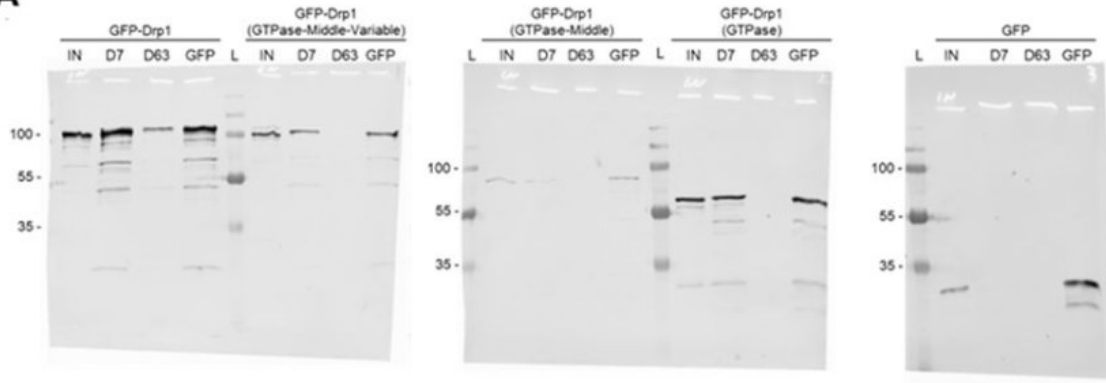
Figure S13.

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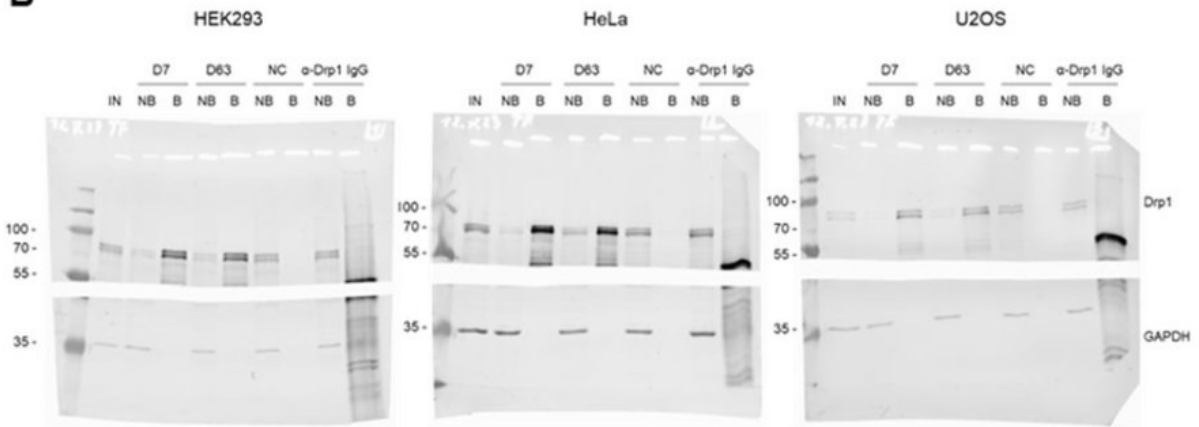
Live cell imaging of Drp1-Cbs in Drp1 KO HeLa cells after treatment with cyanide m-chlorophenylhydrazine.

Time lapse microscopy of Drp1 KO HeLa cells transiently expressing unmodified or turnover-accelerated Drp1-Cbs. 1 d post transfection, cells were stained with Hoechst33258 (blue) and MitoTracker green (green) and subsequently treated with 20 μ M cyanide m-chlorophenylhydrazine. Representative confocal images were taken after 10, 70, and 130 min of treatment. Signal intensity was adjusted for each construct to visualize the residual expression of Drp1-Cbs. Scale bar 25 μ m.

A



B



C

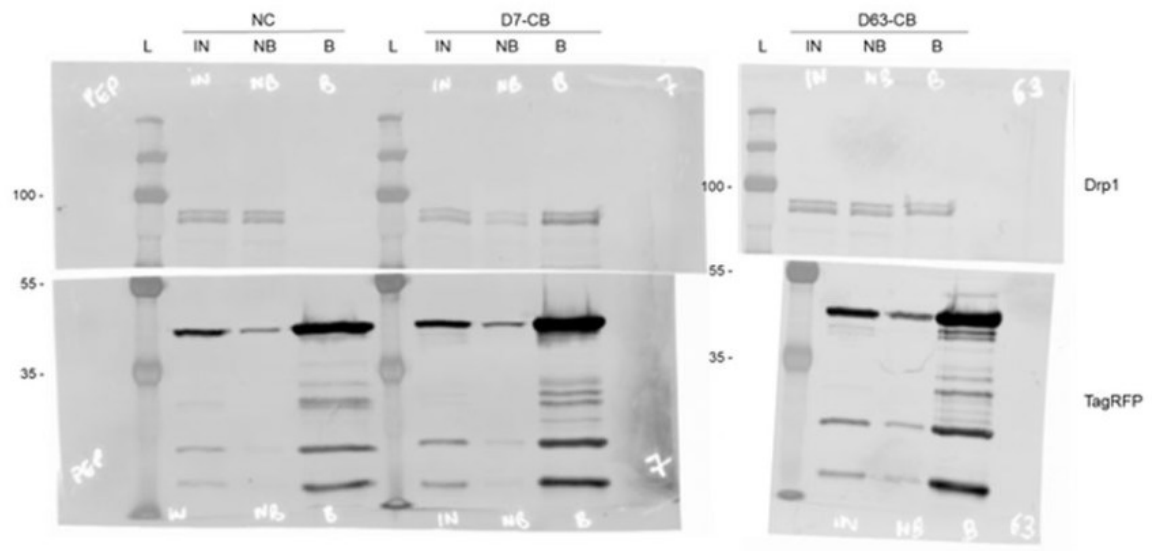


Figure S14.

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Full-size Western Blots images shown in this study.

(A) Full size of Western blots depicted in Fig 2B (domain mapping) stained with an anti-GFP antibody. **(B)** Full size of Western blots depicted in Figs 3A and S3. Top half stained with anti-Drp1 antibody; bottom half stained with anti-GAPDH antibody. **(C)** Full size Western blots depicted in Fig 6A. Top half stained with anti-Drp1 antibody; bottom half stained with anti-TagRFP antibody. Molecular weights in kD are shown on the left of each blot.

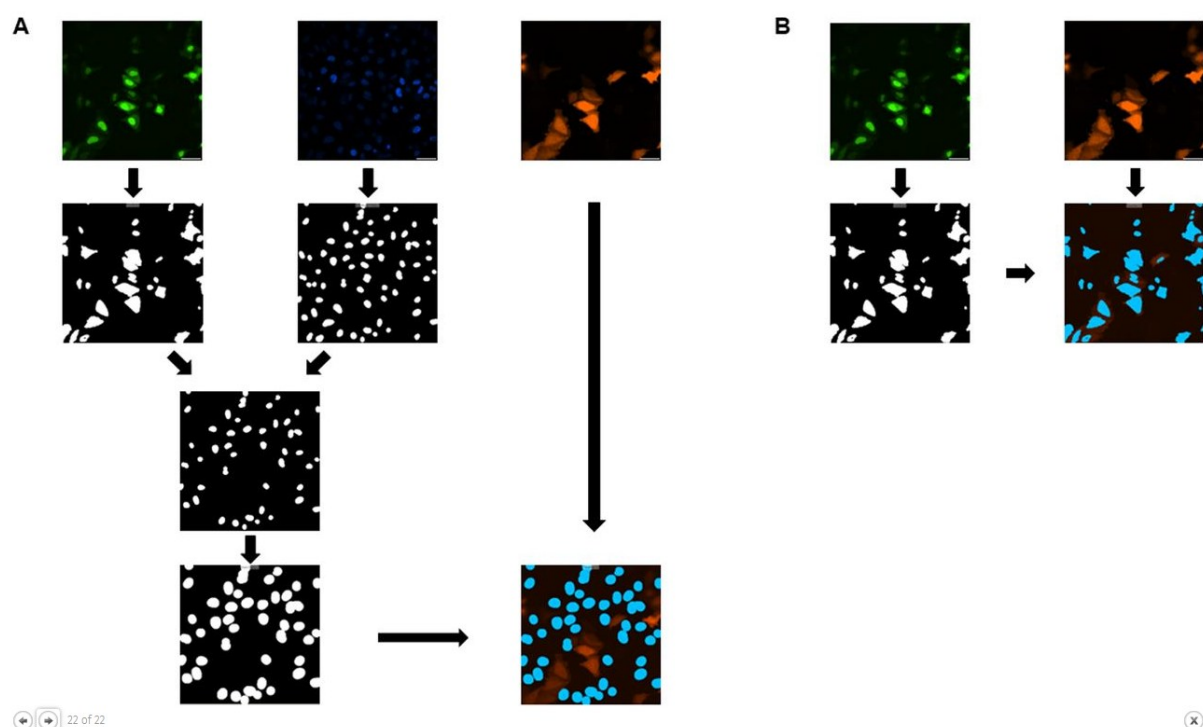


Figure S15.

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Workflow of the automated image segmentation used for Cb signal quantification.

For quantitative image analysis, cells were co-transfected with GFP-NLS (green) and chromobody (Cb, red), and nuclei were stained using DAPI (blue). **(A)** Workflow for background signal determination. Area of GFP-NLS-expressing cells and nuclei were determined individually (second row). Then, nuclei of GFP-NLS-expressing cells were excluded (third row). Next, the DAPI signal was used to create a cell shape and the RFP intensity was measured in this area (forth row). **(B)** Workflow for Cb signal intensity determination. The GFP signal was used to determine the cell shape and the RFP signal was measured in this area.

Table S1  Amino acid sequences of Drp1 Nbs identified as positive clones by Phage ELISA.

Supplementary Table 1:

Drp1 Nb	Amino acid sequence
D7	QVQLVESGGGLVQPGGSLRLSCVASGDFSTYSMTWHRQVLGKERELVASITPGGSRTNVA DSVKGRFTISRDNAMLYLQMDRLKPEDTGMYYCAQGGYYAEPDYWGKGTRVTVSS
D49	EVQLVESGGGWVQPGGSLRLSCSASGFTFRDYVMTWVRQTPGRGLEWVSAIDGSGSKINYL DSVKGRFTISRDNQAMLYLQMNSLRPDDTAVYYCARKGRGGLGEGTYWGKGTPVTVSS
D63	QVQLVESGGDLVQPGGSLKLSCAVSGVSDSLSSFYLMAWHRQAPGKQREMIADIVSGKATY ADSVKGRFTISRDNAAENTAYLQMNSLKPEDTAVYYCNVLIQNRSTFQSYWGQGTQVTVSS
D187	HVQLVESGGGLVQAGESLKLSCVASGIPFSSRAMGWYRQTPGKDRELVARISVRGVTFYADS VTGRFAISRDNDRSTLYLQMNLSKSEDTAIYFCAAGDNTQTVLTPAHWGQGTQVTVSS
D193	EVQLVDSGGGLVQSGGSLTLACAASVSIDEFPAMTWYRQATGKQRELVGVTGKGATKLADS AKGRFTISRDNAMVLYLQMTSLKPDDTAVYYCSVPNKVMGWGPDDWWGQGSQVTVSS
D219	QVQLVESGGGMVQSGGSLSLSCAASGFAFSLYEMSWIRQAQGRGPEWVASINPGSSRTYYT DSVKGRFTISRDNNDKNILYLMNLSKPEDTALYYCARGRTLIGISSDKGSQVTVSS
D246	EVQLVESGGGLAQPGGSLRLSCAASGPTDNIYTMGWYRQPPGQSRQFVASVWVSTGVKAY AKFVGGRFRITKDSAKRTVDLQMDSLQPEDTAIFYCNLNDRVTPMSERDYWGQGTPTVTVSS
D258	EVQLQESGGESVQPGGSLRLSCVVSSTTFSTVAMGWYREVPGKKRELVARISSRGVTFYSD SVEGRFTISKDNDKSTLYLQMNLSLETEDTAMFYCAAAGNSEVVLFRPKYWGQGTPTVTVSS

Table S2  List of significant proteins enriched with the D7 nanotrap from soluble protein fraction of HEK293 cells. List of class A (FDR = 0.01) and class B (FDR = 0.05) interactors of Drp1 precipitated by the D7 nanotrap. -Log(P-value) and difference between D7 and control nanotrap are stated for each protein.

Supplementary Table 2:

Protein	Gene	Class A	Class B	-Log (p-value)	Difference
Dynamin-1-like protein	DNM1L	+		5.67	2.52
Polymerase delta-interacting protein 2	POLDIP2		+	3.95	1.36
Kinesin-like protein KIF15	KIF15		+	5.21	1.35
Cation-dependent mannose-6-phosphate receptor	M6PR		+	3.75	1.17
Transmembrane emp24 domain-containing protein 1	TMED1		+	1.31	0.95
60S ribosomal protein L30	RPL30		+	2.38	0.93
Vesicle-associated membrane protein 3	VAMP3		+	1.29	0.86
Neuroplastin	NPTN		+	4.45	0.85
Caspase-14	CASP14		+	2.66	0.84
Protein CutA	CUTA		+	1.18	0.80
LanC-like protein 1	LANCL1		+	3.35	0.80
LysM and putative peptidoglycan-binding domain-containing protein 2	LYSMD2		+	1.46	0.76
CDGSH iron-sulfur domain-containing protein 1	CISD1		+	3.67	0.73
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1		+	2.81	0.72
Gamma-glutamylcyclotransferase	GGCT		+	1.15	0.72
Microtubule-associated protein 4	MAP4		+	1.67	0.70
DDB1- and CUL4-associated factor 12	DCAF12		+	1.93	0.70
Dynein light chain roadblock-type 1; Dynein light chain roadblock-type 2	DYNLRB1; DYNLRB2		+	2.17	0.69
Hemoglobin subunit beta; Hemoglobin subunit delta	HBB; HBD		+	3.85	0.67
Protein canopy homolog 2	CNPY2		+	1.27	0.66
Mitochondrial import receptor subunit TOM20 homolog	TOMM20		+	2.23	0.66
Histone deacetylase 6	HDAC6		+	2.22	0.65
Protein bicaudal D homolog 2	BICD2		+	4.28	0.64
Sodium-coupled neutral amino acid transporter 2	SLC38A2		+	1.35	0.63
Cytochrome c oxidase subunit 5A	COX5A		+	1.17	0.63
Protein YIPF4	YIPF4		+	2.60	0.62
Lysosome membrane protein 2	SCARB2		+	1.82	0.62
Cytochrome b5 type B	CYB5B		+	1.26	0.62
Synaptogyrin-1	SYNGR1		+	1.41	0.61
DNA damage-binding protein 1	DDB1		+	3.26	0.61
Transmembrane protein 181	TMEM181		+	1.97	0.59
MICOS complex subunit MIC19	CHCHD3		+	2.75	0.59
DDRCK domain-containing protein 1	DDRCK1		+	1.70	0.55
Manganese-transporting ATPase 13A1	ATP13A1		+	2.55	0.54
Coxsackievirus and adenovirus receptor	CXADR		+	1.80	0.53
ATP synthase subunit d, mitochondrial	ATP5H		+	1.97	0.51

Table S3  List of significant proteins enriched with the D63 nanotrap from soluble protein fraction of HEK293 cells List of class A (FDR = 0.01) and class B (FDR = 0.05) interactors of Drp1 precipitated by the D63 nanotrap. -Log(P-value) and difference between D63 and control nanotrap are stated for each protein.

Supplementary Table 3:

Protein	Gene	Class A	Class B	-Log(P-value)	Difference
Dynamin-1-like protein	DNM1L	+		5.61	2.55
Peroxisomal multifunctional enzyme type 2	HSD17B4	+		3.85	1.95
GDP-L-fucose synthase	TSTA3	+		5.71	1.30
Hydroxysteroid dehydrogenase-like protein 2	HSDL2	+		3.11	1.29
Protein CutA	CUTA	+		3.69	1.22
Transmembrane emp24 domain-containing protein 1	TMED1	+		3.39	1.20
Mitochondrial import inner membrane translocase subunit Tim10 B	TIMM10B	+		4.50	1.17
Bifunctional lysine-specific demethylase and histidyl-hydroxylase NO66	NO66		+	2.95	1.05
Cystatin-A	CSTA		+	1.31	0.96
Tight junction protein ZO-1	TJP1		+	3.21	0.92
Importin-11	IPO11		+	1.68	0.92
Exportin-7	XPO7		+	2.87	0.92
Vesicle-associated membrane protein 3	VAMP3		+	1.34	0.87
Caspase-14	CASP14		+	1.09	0.86
60S ribosomal protein L30	RPL30		+	2.24	0.85
E3 ubiquitin-protein ligase TRIM9	TRIM9		+	2.56	0.78
Mitochondrial import receptor subunit TOM20 homolog	TOMM20		+	2.53	0.77
ATP synthase subunit beta	ATP5B		+	1.39	0.76
Apoptosis-inducing factor 1	AIFM1		+	3.21	0.73
CDGSH iron-sulfur domain-containing protein 1	CISD1		+	3.82	0.71
Protein-L-isoaspartate O-methyltransferase domain-containing protein 2	PCMTD2		+	1.88	0.70
Serine/threonine-protein kinase 3	STK3		+	2.83	0.69
STE20/SPS1-related proline-alanine-rich protein kinase	STK39		+	5.23	0.69
LIM domain and actin-binding protein 1	LIMA1		+	1.52	0.68
Adenylate kinase isoenzyme 6	TAF9;AK6		+	3.90	0.67
MICOS complex subunit MIC19	CHCHD3		+	2.91	0.63
Transcription elongation factor B polypeptide 2	TCEB2		+	2.20	0.62
Histone deacetylase 6	HDAC6		+	2.07	0.58
Transcription elongation factor B polypeptide 1	TCEB1		+	2.39	0.57
Protein-L-isoaspartate(D-aspartate) O-methyltransferase	PCMT1		+	3.82	0.57
Ubiquitin carboxyl-terminal hydrolase isozyme L5	UCHL5		+	3.43	0.56
Protein-L-isoaspartate O-methyltransferase domain-containing protein 1	PCMTD1		+	3.03	0.55

Table S4  List of significant proteins enriched with the D7 and D63 nanotraps from soluble protein fraction of HeLa cells.

Supplementary Table 4:

Protein	Gene	Class A	Class B	-Log(P-value)	Difference
D7					
Dynamin-1-like protein	DNM1L	+		4,40	3,31
Dynamin-1-like protein	DNM1L	+		4,55	2,34
Polymerase delta-interacting protein 2	POLDIP2		+	4,52	1,17
Kinesin-like protein KIF15	KIF15		+	4,99	1,86
D63					
Dynamin-1-like protein	DNM1L	+		4,28	3,09
Dynamin-1-like protein	DNM1L		+	2,98	0,92
Dynamin-1-like protein	DNM1L		+	3,55	1,95
Kelch-like protein 13;Kelch-like protein 9	KLHL13;KLHL9		+	3,35	1,09
GDP-L-fucose synthase	TSTA3		+	4,53	1,11
Protein-L-isoaspartate O-methyltransferase domain-containing protein 1	PCMTD1		+	4,34	1,24
Mitochondrial import inner membrane translocase subunit Tim10 B	TIMM10B		+	2,84	1,03

Table S5  List of significant proteins enriched with the D7 and D63 nanotraps from soluble protein fraction of U2OS cells.

Supplementary Table 5

Protein	Gene	Class A	Class B	-Log(P-value)	Difference
D7					
Dynamin-1-like protein	DNM1L		+	3.69	3.02
Dynamin-1-like protein	DNM1L		+	3.33	1.37
Polymerase delta-interacting protein 2	POLDIP2		+	3,642	1,31
Kinesin-like protein KIF15	KIF15		+	3,35	1,27
D63					
Dynamin-1-like protein	DNM1L		+	3,63	2,62
GDP-L-fucose synthase	TSTA3		+	5,00	0,90
Enhancer of mRNA-decapping protein 3	EDC3		+	4.50	0.82
Kelch-like protein 13;Kelch-like protein 9	KLHL13;KLHL9		+	3.35	1.09
Lymphokine-activated killer T-cell-originated protein kinase	PBK		+	4.50	0.78

Table S6  List of oligonucleotides used in this study.

Supplementary Table 6:

Name	Sequence 5'-3'	purpose
CALL001	GTCCTGGCTGCTCTTCTACAAGG	Nb library generation
CALL002	GGTACGTGCTGTTGAACTGTTCC	
FR1-1	CATGGCNSANGTGCAGCTGGTGGANTCNGGNGG	
FR1-2	CATGGCNSANGTGCAGCTGCAGGANTCNGGNGG	
FR1-3	CATGGCNSANGTGCAGCTGGTGGANAGYGGNGG	
FR1-4	CATGGCNSANGTGCAGCTGCAGGANAGYGGNGG	
FR1-ext1	GTAGGCCAGCCGCGCCATGGCNSANGTGCAGCTGGTGG	
FR1-ext2	GTAGGCCAGCCGCGCCATGGCNSANGTGCAGCTGCAGGA	
FR4-1	GATGCGGCCGCGNANGANACGGTGACCNGNRYNCC	
FR4-2	GATGCGGCCGCGNANGANACGGTGACCNGNGANCC	
FR4-3	GATGCGGCCGCGNANGANACGGTGACCNCRCTNCC	
FR4-4	GATGCGGCCGCRCTNANGANACGGTGACCNGNRYNCC	
FR4-5	GATGCGGCCGCRCTNANGANACGGTGACCNGNGANCC	
FR4-6	GATGCGGCCGCRCTNANGANACGGTGACCNCRCTNCC	
bivD7-1	GAAACGTCTCAACTCTCAGGTGCAGCTGGTGGAGAG	Cloning of bivNbs
bivD7-2	CACCACCGCCAGATCCACCGCCACCTGATCCTCCGCCTCCTGAGGAGACGGTGACCCG	
bivD7-3	GGTGGATCTGGCGGTGGTGAAGTGGTGGCGGAGGTAGTCAGGTGCAGCTGTGGAGAG	
downEcoRI-rev	GTTGTAAAACGACGGCCAGTG	
bivNshort_for	GAAACGTCTCAACTCTCAGGTGC	
bivD63forN	GAAACGTCTCAACTCTCAAGTGCAGCTGGTGGAGTC	
bivNtermGS-rev	CACCACCGCCAGATCCACCGCCACCTGATCCTCCGCCTCCGCTGCTAACGGTAACCTG	
bivD63forC	GGTGGATCTGGCGGTGGTGAAGTGGTGGCGGAGGTAGTCAAGTGCAGCTGTGGAGTC	
bivD63_fusion	GAAACGTCTCAACTCTCAAGTGC	GFP-Drp1 generation
Drp1_GTPaseFor	GTGGTGGCGGAGGTAGCATGGAGGCGCTAATTCCTGTC	
Drp1_C_term_rev	CTAGATCCGGTGGATCCTCACCAGAGATGAGTCTCCCG	
Drp1_VD_rev	CTAGATCCGGTGGATCCTCATTTTCGTGCAACAGGAAC	generation of domain deletion constructs
Drp1_MD_rev	CTAGATCCGGTGGATCCTCAAAAGTCTGGATGTTTTGTG	
Drp1_GTPaseRev	CTAGATCCGGTGGATCCTCATTTGAGTAAAGTAGCACTTTTATC	

Table S7  List of expression constructs used in this study.

Supplementary Table 7:

construct	origin
pHEN4	(Arbabi Ghahroudi M et al, 1997)
pHEN6C	(Rothbauer U et al, 2008)
D7 in pHEN6C	This study
D63 in pHEN6C	This study
pCDNA3.4	Addgene
bivD7 in pCDNA3.4	This study
bivD63 in pCDNA3.4	This study
pTagRFP	(Panza P et al, 2015)
D7 in pTagRFP	This study
D63 in pTagRFP	This study
pEGFP-Ubi-R-3xGS-tagRFP	(Keller BM et al, 2018)
D7 in pEGFP-Ubi-R-3xGS-tagRFP	This study
D63 in pEGFP-Ubi-R-3xGS-tagRFP	This study
AcGFP-Drp1	(Jenner A et al, 2022)
pEGFP-N1	Clontech
Drp1 in pEGFP-N1	This study
GTPase-MD-VD in pEGFP-N1	This study
GTPase-MD in pEGFP-N1	This study
GTPase in pEGFP-N1	This study
pEGFP-C1	Clontech
Pep-Cb in pTagRFP	(Traenkle B et al, 2020)
BC2T-GFP-NLS in pEGFPN1	(Virant D et al, 2018)
pTYB2	(Jenner A et al, 2022)

Table S8  List of antibodies used in this study.

Supplementary Table 8:

target	animal	clone	conjugation	manufacturer	catalog number
Drp1	rabbit	D6C7	-	Cell Signaling Technology	#8570
GAPDH	rabbit	polyclonal	-	Invitrogen	PA1-987
GFP	rat	3H9	-	ChromoTek	-
TagRFP	rabbit	polyclonal	-	Evrogen	AB234
M13	mouse	monoclonal	HRP	GE Healthcare	27-9421-01
rabbit	donkey	polyclonal	AF647	Invitrogen	#A-31573
rabbit	goat	polyclonal	AF488	Invitrogen	#A-11008
rat	donkey	polyclonal	AF647	Invitrogen	#A-21247
alpaca	goat	polyclonal	Cy5	Jackson Immunoresearch	128-175-232

10 Appendix II

A Nanobody-Based Toolset to Monitor and Modify the Mitochondrial GTPase Miro1

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A Nanobody-Based Toolset to Monitor and Modify the Mitochondrial GTPase Miro1

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The mitochondrial outer membrane (MOM)-anchored GTPase Miro1, is a central player in mitochondrial transport and homeostasis. The dysregulation of Miro1 in amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) suggests that Miro1 may be a potential biomarker or drug target in neuronal disorders. However, the molecular functionality of Miro1 under (patho-) physiological conditions is poorly known. For a more comprehensive understanding of the molecular functions of Miro1, we have developed Miro1-specific nanobodies (Nbs) as novel research tools. We identified seven Nbs that bind either the N- or C-terminal GTPase domain of Miro1 and demonstrate their application as research tools for proteomic and imaging approaches. To visualize the dynamics of Miro1 in real time, we selected intracellularly functional Nbs, which we reformat into chromobodies (Cbs) for time-lapse imaging of Miro1. By genetic fusion to an Fbox domain, these Nbs were further converted into Miro1-specific degrons and applied for targeted degradation of Miro1 in live cells. In summary, this study presents a collection of novel Nbs that serve as a toolkit for advanced biochemical and intracellular studies and modulations of Miro1, thereby contributing to the understanding of the functional role of Miro1 in disease-derived model systems.

Keywords: Miro1, nanobodies, imaging, proteomics, degron

INTRODUCTION

Neurodegenerative disorders including Alzheimer's disease, Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) pose a major public health challenge especially in societies with a rapidly aging population. Due to the fundamental role of mitochondria in energy production, calcium homeostasis, reactive oxygen species (ROS) formation, and initiation of apoptosis (Duchen, 2004), pathological mitochondrial morphologies and a dysfunctional quality control are among the main drivers in the development and progression of neurodegeneration (Wang et al., 2019; Monzio Compagnoni et al., 2020). Within cells, mitochondria are continuously transported along actin- and microtubule-based cytoskeletal pathways to areas in demand of ATP supply or calcium buffering (Rube and Van Der Bliek, 2004; Reis et al., 2009). Microtubule-based transport is effected by the motor proteins kinesins and dyneins in anterograde and retrograde directions, respectively (Boldogh

and Pon, 2007). Miro1, a member of the Rho GTPase family, which is mainly located on the surface of the mitochondrial outer membrane (MOM) (Boureaux et al., 2007; Yamaoka and Hara-Nishimura, 2014; Kay et al., 2018), acts as an adaptor to tether motor complexes to mitochondria. Structurally, Miro1 is composed of two distinct N- and C-terminally located GTPase domains flanking a pair of calcium ion (Ca^{2+}) binding EF-hands and a C-terminal transmembrane domain anchored in the MOM (Fransson et al., 2003; Fransson et al., 2006). By its cytoplasmic domains, Miro1 interacts with the adaptor proteins TRAK1 and TRAK2 (Fransson et al., 2006), which recruit the motor proteins kinesin-1 (KIF5B) and dynein/dynactin to facilitate mitochondrial transport along microtubules (MacAskill et al., 2009a; van Spronsen et al., 2013). This motor/adaptor complex is regulated by Ca^{2+} levels. At high concentrations, Ca^{2+} arrests mitochondrial transport by binding to the EF hand domains of Miro1, causing the motor complex to detach from the organelle (Saotome et al., 2008; MacAskill et al., 2009b; Wang and Schwarz, 2009; Schwarz, 2013). Similarly, Miro1 interacts with Cenp-F for cell cycle-dependent distribution of mitochondria during cytokinesis (Kanfer et al., 2015; Okumoto et al., 2018), and overexpression of Miro1 was shown to enhance intercellular transfer of mitochondria from mesenchymal stem cells to stressed epithelial cells (Ahmad et al., 2014; Babenko et al., 2018). Besides mitochondria, Miro1 was also shown to play an important role in peroxisomal transport (Castro et al., 2018; Okumoto et al., 2018). Additionally, Miro1 is a known substrate of the mitophagy-associated PINK1/Parkin quality control system (Wang et al., 2011). Impaired Miro1 ubiquitination has been recently linked to Parkin mutants found in Parkinson's disease (PD) patients and in fibroblasts from an at-risk cohort (Hsieh et al., 2016; Nguyen et al., 2021). This was further confirmed by the identification of mutations in the Miro1 gene *RHOT1* causing an altered mitophagy response (Grossmann et al., 2019; Grossmann et al., 2020). Although not yet demonstrated at the molecular level, dysregulated cellular levels of Miro1 have been described in ALS animal models and in patients (Zhang et al., 2015; Moller et al., 2017). These results, in combination with recent data describing aberrant peroxisomal metabolism in PD patients (Dragonas et al., 2009; Grossmann et al., 2020), strongly suggest a multifactorial link between Miro1 and neurological diseases. Consequently, Miro1 is considered as an emerging biomarker and potential drug target in neuropathology (Hsieh et al., 2019; Nguyen et al., 2021).

Despite *in vitro* and *in vivo* studies already performed on Miro1, detailed information on its structure-related function and cellular dynamics is still lacking, which may be due to the limitation of currently available research tools for Miro1. Here, the use of single-domain antibody fragments, also known as nanobodies (Nbs) derived from heavy chain-only antibodies found in camelids or sharks (Hamers-Casterman et al., 1993; Greenberg et al., 1995), could be advantageous due to their unique binding properties and versatile formats. During the last decade Nbs have been established as attractive alternatives to conventional antibodies for a multitude of biochemical assays (Rothbauer et al., 2008; Cheloha et al., 2020; He et al., 2021; Wagner et al., 2021) and advanced imaging applications

[reviewed in (Traenkle and Rothbauer, 2017; Hrynchak et al., 2021; Naidoo and Chuturgoon, 2021)]. Additionally, their small size, high solubility and stability qualify Nbs for intracellular expression [reviewed in (Helma et al., 2015; Wagner and Rothbauer, 2020)]. Intracellularly functional Nbs genetically fused to fluorescent proteins, designated as chromobodies (Cbs), have been successfully applied for tracing their target antigens in different cellular compartments as well as in whole organisms [reviewed in (Traenkle and Rothbauer, 2017; Wagner and Rothbauer, 2021)]. For the generation of advanced research tools to study Miro1, we have developed specific Nbs derived from a Nb gene library of an immunized animal. Following an in depth characterization of their binding properties, we selected candidates applicable as capture and detection reagents in biochemical assays, immunofluorescence staining and live-cell imaging. Additionally, we designed specific degrons for selective degradation of Miro1 within living cells. Based on our results, we propose that the presented Nb-based toolkit opens new opportunities for more comprehensive studies of Miro1 and helps to elucidate its multifaceted roles for mitochondrial malfunction in neurological disorders.

RESULTS

Identification of Miro1-Specific Nbs

To generate Nbs specific for human Miro1 (Miro1), we immunized an alpaca (*Vicugna pacos*) with recombinant Miro1 adopting a 91-day immunization protocol. After confirmation of a successful immune response in a serum ELISA (Supplementary Figure S1), we generated a phagemid library of $\sim 1.5 \times 10^7$ clones from peripheral B lymphocytes of the immunized animal, representing the diverse repertoire of variable domains (VHs or Nbs) of the heavy chain-only antibodies. For the selection of Miro1-specific Nbs, we performed phage display using either bacterially expressed Miro1 passively adsorbed to immunotubes or site-directed immobilized GFP-Miro1 from HEK293 cells, which was captured in multiwell plates precoated with the GFP-Nb (Supplementary Figure S2A). After two and three rounds of biopanning against Miro1 or GFP-Miro1, we analysed a total of 200 individual clones by phage ELISA and identified 42 positive binders (Supplementary Figures S2B,S2C). Sequence analysis revealed seven unique Nbs with highly diverse complementarity determining regions (CDR) 3 (Figure 1A, Supplementary Table S2). For further analysis, Miro1-Nbs were produced with a C-terminal His₆ tag in *Escherichia coli* (*E.coli*) and purified *via* immobilized metal ion affinity chromatography (IMAC) followed by size exclusion chromatography (SEC) (Figure 1B). Binding affinities were assessed by biolayer interferometry (BLI) for which we immobilized biotinylated Nbs on streptavidin (SA) biosensors and measured their binding kinetics by loading different concentrations of Miro1. Our results showed that all seven Nbs bind Miro1 with high affinities in the low nanomolar range with K_D values ranging from 1.9 to 29.5 nM (Figure 1C; Table 1, Supplementary Figures S3A–S3F).

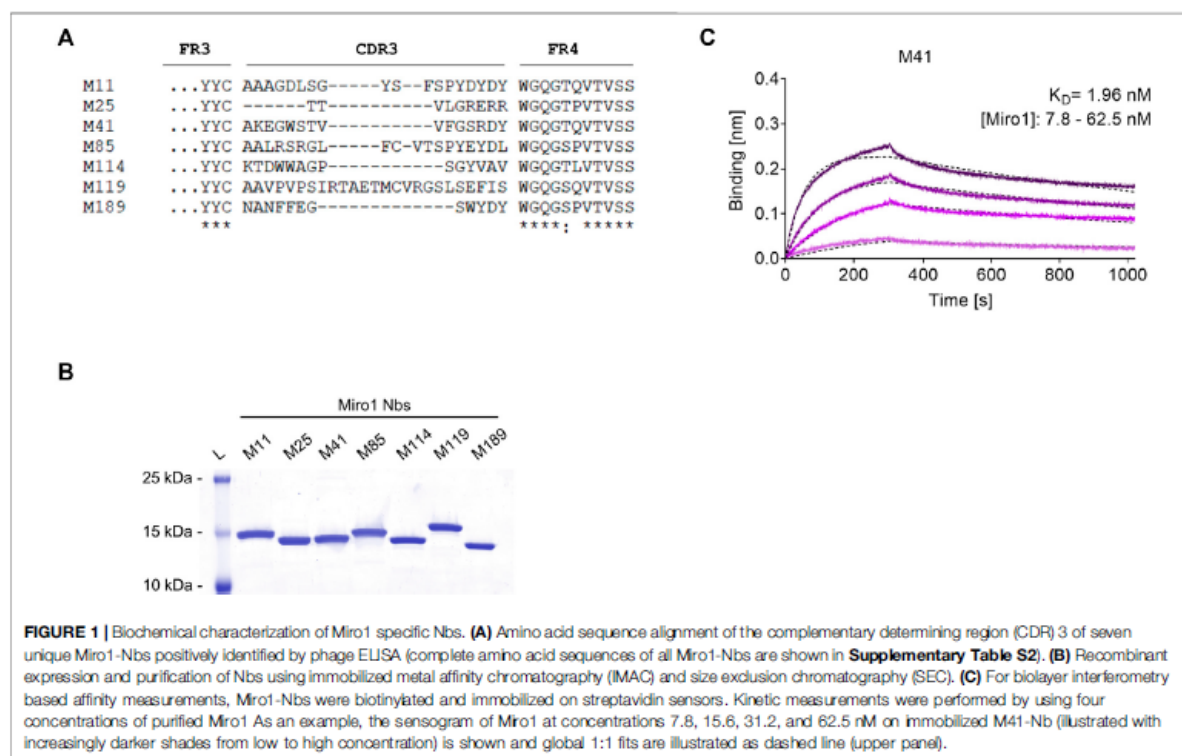


FIGURE 1 | Biochemical characterization of Miro1 specific Nbs. **(A)** Amino acid sequence alignment of the complementary determining region (CDR) 3 of seven unique Miro1-Nbs positively identified by phage ELISA (complete amino acid sequences of all Miro1-Nbs are shown in **Supplementary Table S2**). **(B)** Recombinant expression and purification of Nbs using immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). **(C)** For biolayer interferometry based affinity measurements, Miro1-Nbs were biotinylated and immobilized on streptavidin sensors. Kinetic measurements were performed by using four concentrations of purified Miro1. As an example, the sensogram of Miro1 at concentrations 7.8, 15.6, 31.2, and 62.5 nM on immobilized M41-Nb (illustrated with increasingly darker shades from low to high concentration) is shown and global 1:1 fits are illustrated as dashed line (upper panel).

TABLE 1 | Summary of affinities (K_D), association (k_{ON}) and dissociation constants (k_{OFF}) and the coefficient of determination (R^2) determined for Miro1-Nbs by BLI.

Nanobody	K_D (nM)	k_{ON} ($10^5 \text{ M}^{-1} \text{ s}^{-1}$)	k_{OFF} (10^{-4} s^{-1})	R^2
M11	3.34	2.55 ± 0.02	8.54 ± 0.03	0.97
M25	29.50	1.36 ± 0.04	0.40 ± 0.28	0.80
M41	1.96	3.01 ± 0.01	5.89 ± 0.02	0.99
M85	7.75	1.00 ± 0.01	7.79 ± 0.02	0.99
M114	4.61	0.88 ± 0.01	4.04 ± 0.01	0.99
M119	2.80	2.25 ± 0.01	6.31 ± 0.02	0.99
M189	2.99	1.70 ± 0.01	5.07 ± 0.02	0.98

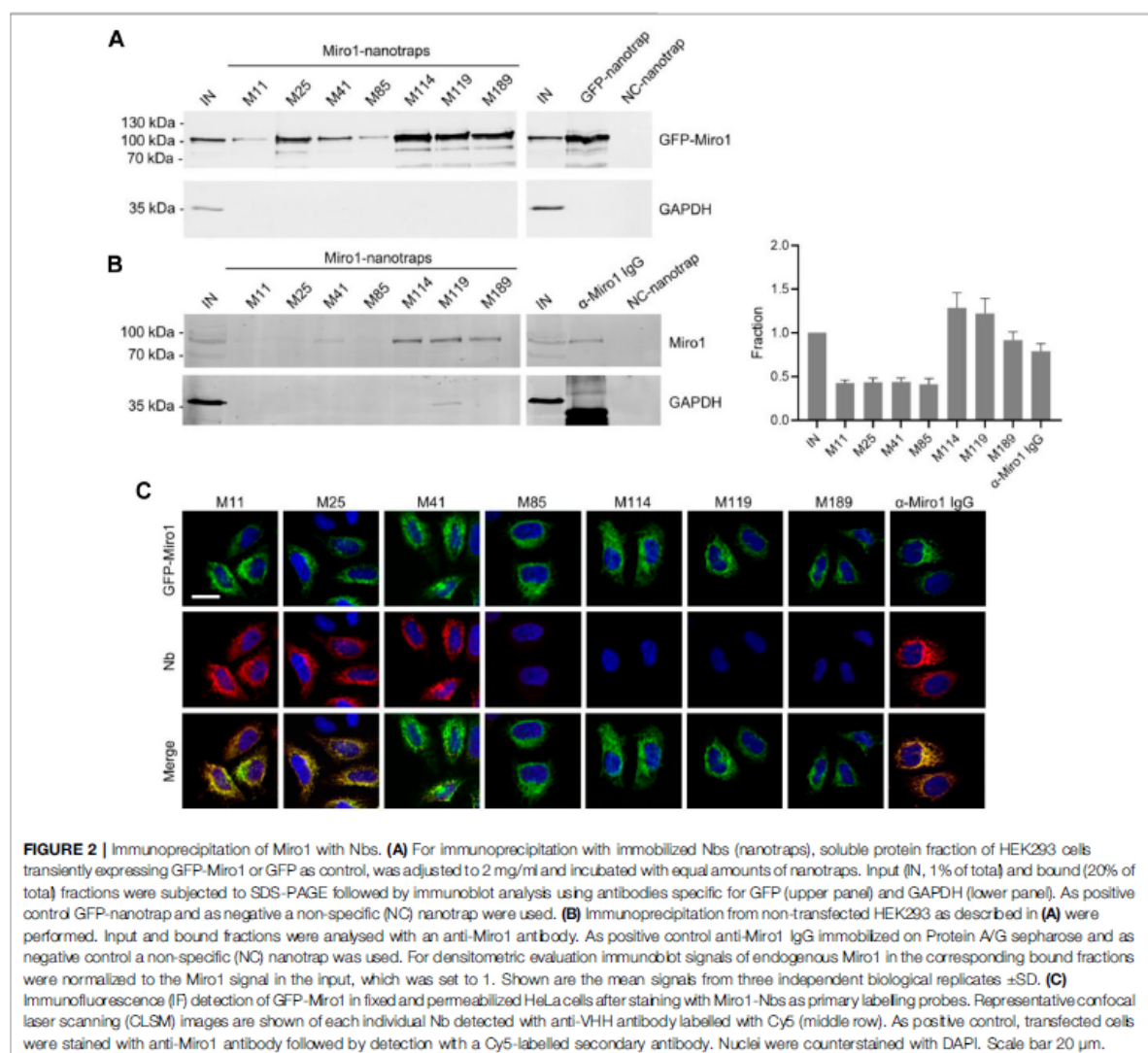
Immobilized Miro1-Nbs Specifically Precipitate Miro1

Considering that a variety of Nbs covalently immobilized to solid matrices such as agarose particles have been applied as pull-down reagents to capture their antigens (Rothbauer et al., 2008; Maier et al., 2015; Braun et al., 2016), we used this approach to investigate the functionality of Miro1-Nb candidates for immunoprecipitation (IP). We chemically coupled Miro1-Nbs to N-hydroxysuccinimide (NHS)-activated agarose particles thereby generating Miro1-nanotrap. First, these nanotrap were incubated with the soluble fraction of cell lysates derived from HEK293 cells expressing GFP-Miro1 or GFP as control. Immunoblot analysis of input and bound fractions revealed that all Miro1-nanotrap except M11 and M85 efficiently precipitated GFP-Miro1, with M114 and M119 exhibiting the highest pull-down efficiencies comparable to the

commercially available high-affinity GFP nanotrap (Rothbauer et al., 2008) (ChromoTek) (Figure 2A). Notably, none of the tested nanotrap showed unspecific binding to GFP or GAPDH used as an endogenous control (Figure 2A, Supplementary Figure S4). Next, we investigated the potential of the nanotrap to precipitate also endogenous Miro1. Quantitative immunoblot analysis revealed Miro1 in the bound fractions of M41, M114, M119, and M189. The levels of precipitated Miro1 were comparable or slightly higher to those obtained with a conventional anti-Miro1 antibody, while no unspecific binding of Miro1 to a nanotrap displaying a non-related Nb was detected (Figure 2B). These findings showed, that at least four of our selected Miro1-Nbs are able to bind their epitopes within endogenous Miro1 under the tested lysis conditions. However, since we observed rather low levels of endogenous Miro1 in the input and in the bound fractions, we tested different cell types and different lysis conditions (Supplementary Figures S5A–S5C). Results from these experiments showed that even upon lysis with the partially denaturing RIPA buffer only minor fractions of endogenous Miro1 are transferred to the soluble protein fraction, making it difficult to study Miro1 regardless of the capturing reagent used.

Immunofluorescence Studies With Miro1-Nbs

For a second functional testing, we examined the performance of Miro1-Nbs in immunofluorescence (IF). Therefore we applied

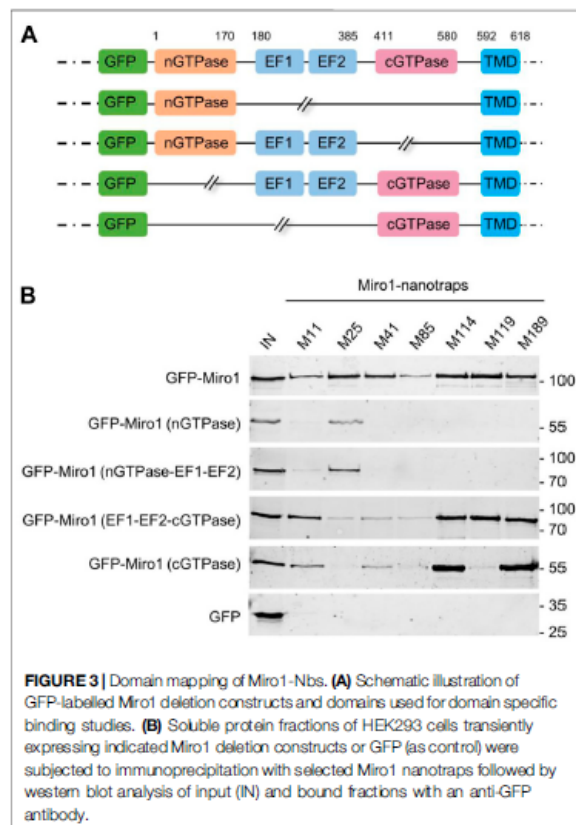


the Nbs as primary binding molecules in combination with fluorescently labelled anti-VHH antibodies in fixed and permeabilized HeLa cells transiently expressing GFP-Miro1. Interestingly, Nbs M11 and M25, which did not capture Miro1 by immunoprecipitation showed a clear co-localization with GFP-Miro1 at mitochondrial structures. It is possible that these Nbs recognize epitopes that were not accessible or structurally altered under the lysis conditions used for immunoprecipitation. However, with the M41-Nb, we identified one candidate that showed functionality in both assays (Figure 2C). When we tested the Miro1-Nbs for immunofluorescence staining of endogenous Miro1, we did not detect clear colocalization with mitochondria as observed with an anti-Miro1 antibody (data not shown). At this point, we can only speculate about the causes. It is possible that the epitopes

of endogenous Miro1 are blocked by binding partners or that structural changes occurred due to the permeabilization and fixation procedures. However, it is also conceivable that the affinities of the Miro1-Nbs are not sufficient to act as primary binding molecules in immunofluorescence.

Selected Miro1-Nbs Bind Different Domains of Miro1

To generate well-characterized binders, detailed knowledge of their recognized epitopes or domains is mandatory. Considering that Nbs preferentially bind conformational epitopes (De Genst et al., 2006; Pardon et al., 2014), we performed domain mapping to identify the binding regions recognized by our Miro1-Nbs. Therefore, we generated a series of Miro1 domain deletion



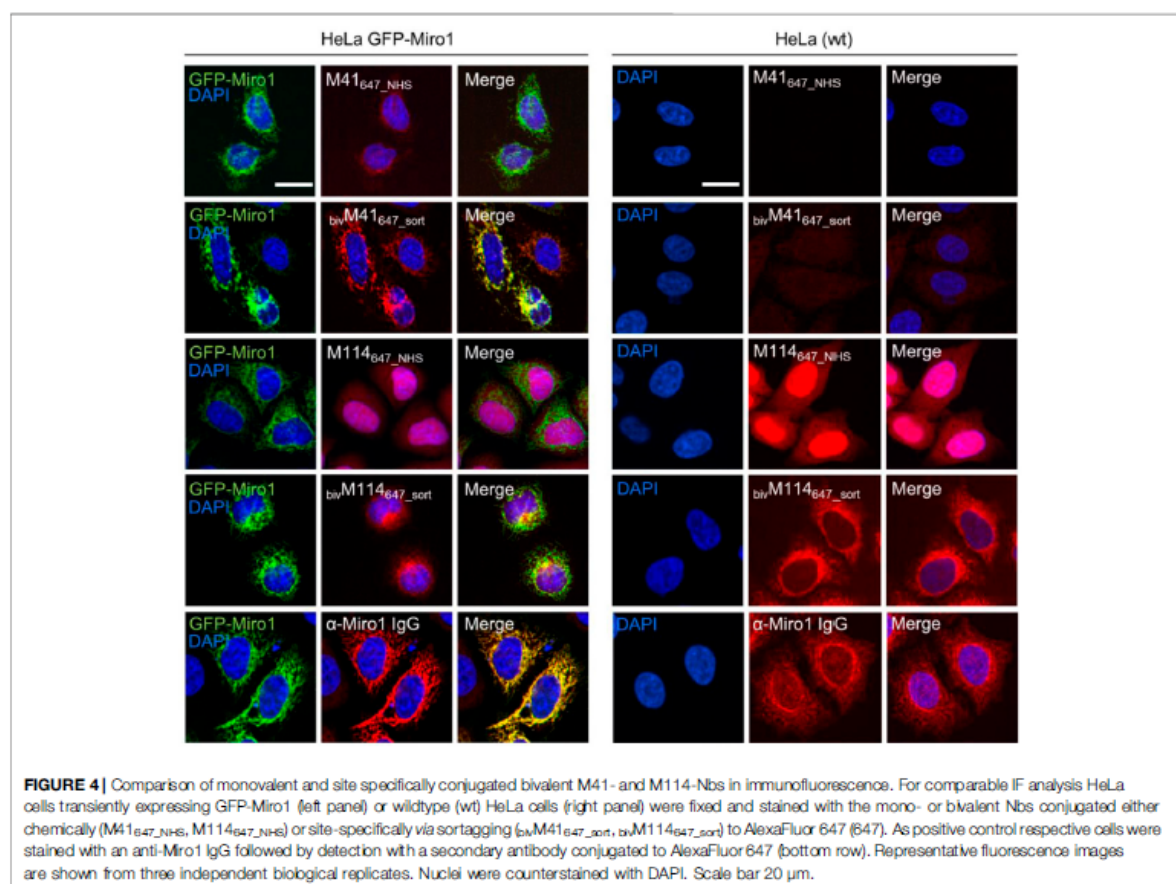
constructs fused C-terminally to GFP (Figure 3A) and performed pulldown studies upon expression of these deletion constructs in HEK293 cells as described above. Analysis of the bound fractions revealed that M11, M41, M85, M114, and M189 specifically recognize epitopes within the C-terminal GTPase domain while M25 addresses an epitope spanning the N-terminal GTPase in combination with the EF-hand domains. For M119, we observed interactions involving regions of the EF hands as well as the C-terminal GTPase domains (Figure 3B, Supplementary Figure S6).

Optimized, Bivalent Nbs Show Improved Capture and Detection of Miro1

With M41- and M114-Nb we identified two candidates that have high affinities, are devoid of additional disulfide bonds and tested positive in IP and/or IF detection of Miro1. In the monovalent format, however, both Nbs bind only small amounts of Miro1. To improve their binding performance, we genetically fused the coding sequences of two M41- or two M114-Nbs head-to-tail, inserting a flexible Gly-Ser linker [(G₄S)₄] of 20 amino acids and generated a bivalent M41-Nb (bivM41) and a bivalent M114-Nb (bivM114). Following production and purification from mammalian cells (Supplementary Figure S7A), we analyzed

their binding kinetics by BLI measurements showing similar or slightly improved affinities (Supplementary Figure S7B). To avoid potential reduction in binding due to nonspecific modification of lysine residues by NHS-based coupling, we changed to a site-specific functionalization strategy. Thus, we introduced an azide-modified peptide at the C-terminus of the bivalent Nbs via chemoenzymatic sortagging (Popp and Ploegh, 2011; Virant et al., 2018) followed by click-chemistry addition of a dibenzocyclooctyne (DBCO) derivative. This enabled us to flexibly conjugate either agarose particles or fluorescent dyes specifically to the C-terminus of the bivalent Nbs (Jewett and Bertozzi, 2010). With this approach, we converted bivM41 and bivM114 into fluorescently-labelled bivalent imaging probes and nanotraps, which we further tested in their respective applications.

First, we performed IF staining of HeLa cells with either the monovalent Nbs, which were chemically coupled to AlexaFluor (AF) 647 (M41_{647_NHS}; M114_{647_NHS}) or the bivalent formats, which were C-terminally conjugated to AF647 (bivM41_{647_sort}; bivM114_{647_sort}). For bivM41_{647_sort} image analysis showed a significantly improved staining and a crisp overlap of the GFP-Miro1 and Nb signal at mitochondrial structures compared to the monovalent version. Notably, when tested for detection of endogenous Miro1 in HeLa cells, bivM114_{647_sort} also shows a strong Miro1 staining at mitochondrial structures (Figure 4). Next, we tested the different nanotraps to pull down endogenous Miro1. Comparative immunoprecipitation of endogenous Miro1 from lysates of HEK293 cells using either the chemically immobilized monovalent nanotraps (M41_{NHS}; M114_{NHS}) or the site-directed modified versions (bivM41_{sort}; bivM114_{sort}) revealed a considerably increased accumulation of endogenous Miro1 after pulldown with bivM41_{sort} and also slightly higher enrichment for bivM114_{sort}. In both cases the amount of precipitated Miro1 was higher compared with the conventional Miro1 antibody used as a positive control (Figure 5A). For a more detailed analysis, we continued and performed an in-depth comparison of the mono- and bivalent nanotraps (M41_{NHS}; M114_{NHS} and bivM41_{sort}; bivM114_{sort}) using mass-spectrometry analysis to evaluate their potential use in protein interaction studies. In total, three technical replicates for each nanotrap were performed, with equal cell number as input material, which allowed us to apply label-free quantification. Initially, we validated the reproducibility between replicates, which showed a Spearman rank correlation close to one (Supplementary Figure S8A). Correlation was lower between the different monovalent or bivalent nanotraps, suggesting a difference in their performance. These findings were further supported by principal component analysis (PCA), for which we observed that ~66% of the sample variance in the first component can be explained by the two different nanotrap formats used (Supplementary Figure S8B). Next, we analysed the efficiency of each nanotrap to capture endogenous Miro1. Sequence alignment analysis of the eight annotated Miro1 isoforms showed a high level of sequence identity between isoforms (data not shown). In total, 31 “razor” peptides were identified for Miro1 isoform 3 and 30 peptides, including one unique peptide, for isoform 2 (Supplementary Figure S8C). Both bivalent nanotraps, as well



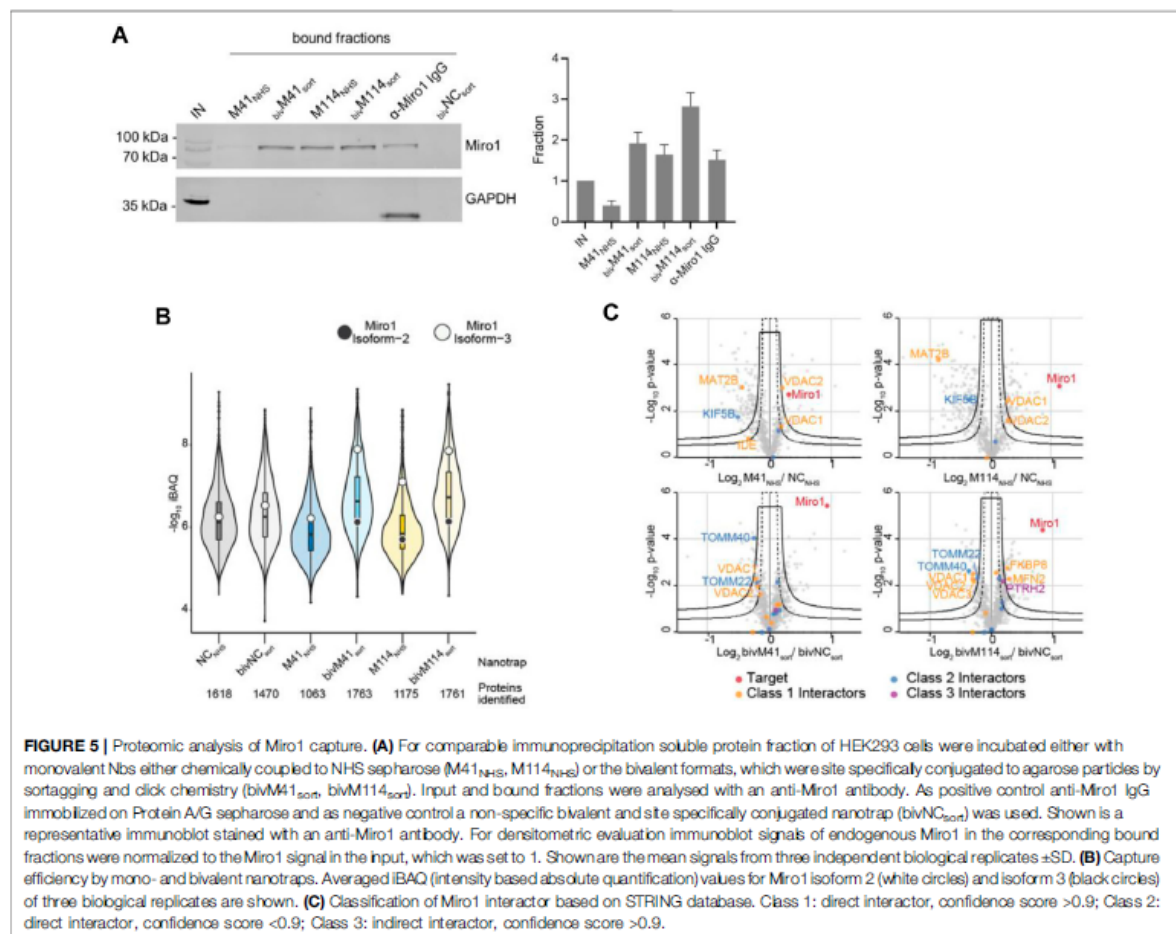
as the M114_{NHS} nanotrapp, were able to capture isoform 2 in addition to isoform 3. Notably, bivalent nanotraps allowed for the identification of more Miro1 peptides compared to their monovalent formats, which is also reflected by the higher Miro1 sequence coverage (Supplementary Figure S8D). We validated the enrichment efficiency of Miro1 capture based on intensity based absolute quantification (iBAQ) (Figure 5B). Overall, we detected a higher background for bivalent nanotraps of up to 1,760 proteins, which is comparable to the controls. Despite the high background, the highest level of Miro1 isoform 2 and 3 was detected for both bivalent nanotraps. While the M41_{NHS} nanotrapp showed comparable levels of Miro1 as the controls, M114_{NHS} as well as the bivalent nanotraps all showed an increased Miro1 capture. Notably, our MS analysis revealed no enrichment of structurally related proteins of the Ras GTPase family (Supplementary Table S3). Finally, we classified known Miro1 interactors (based on STRING database annotation), by their direct or indirect interaction with Miro1 in combination with the confidence score. While all nanotraps showed a clear enrichment of Miro1, in comparison to the control nanotraps, only the bivM114_{sort}

allowed for the enrichment of class 1 interactors such as MFN2 or FKBP8 (Figure 5C).

In summary, these results demonstrate how the performance of Nbs as capture and detection tools can be improved by generating bivalent binding molecules in combination with site-specific functionalization. From our data we concluded that the bivalent Miro1-Nbs have a high potential as detection probes to visualize even low levels of endogenous Miro1. Similarly, the bivalent nanotraps showed an improved performance in capturing Miro1. Especially the site-specifically modified bivM114-Nb might be a suitable capture reagent to be applied in future interactome studies of Miro1.

Characterization of Intracellular Binding of Miro1-Cbs

The advantage of Cbs, defined as chimeric expression constructs comprising a Nb genetically fused to a fluorescent protein, is that they can visualize dynamic redistribution and expression levels of endogenous antigens within living cells with spatial and temporal resolution [reviewed in (Wagner and Rothbauer, 2021)]. To analyse the functionality of selected Miro1-Nbs for

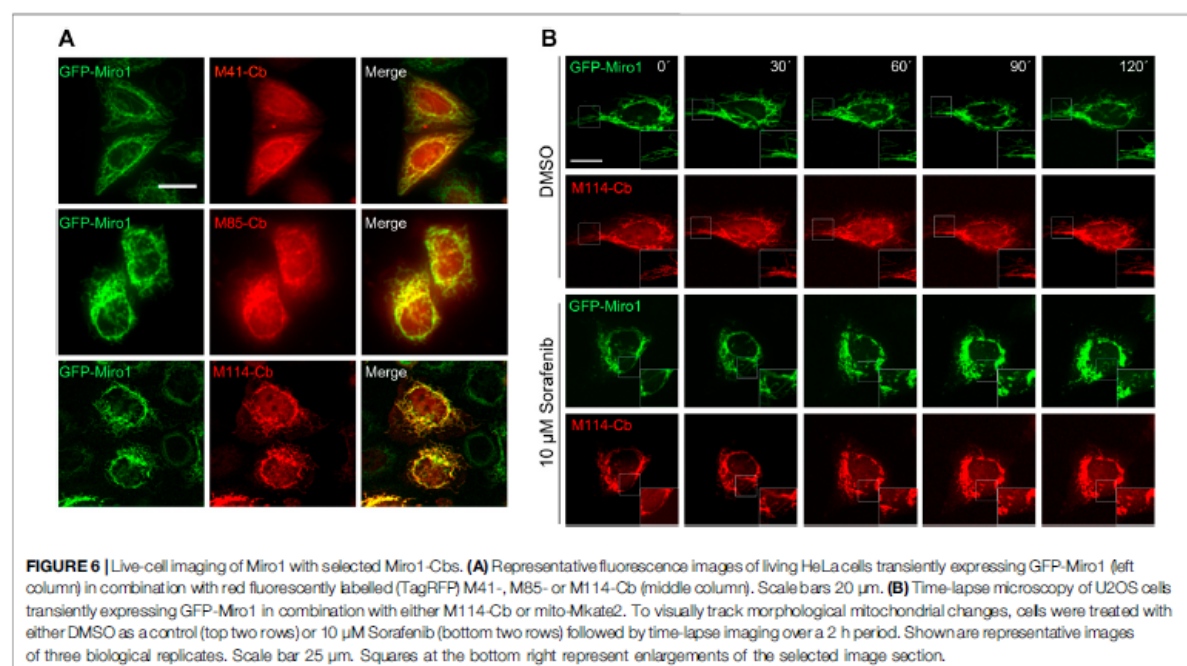


intracellular targeting and tracing of Miro1 in living cells, we converted them into Cbs by fusing the Nb-coding sequences *via* a flexible GS linker to TagRFP. The Cb constructs were transiently expressed either alone or in combination with GFP-Miro1 in HeLa cells followed by live-cell fluorescence imaging. For M41-, M85- and M114-Cb, we observed a clear relocalization of the Cb signal to mitochondrial networks in the presence of GFP-Miro1 (Figure 6A), whereas all other Miro1-Cbs did not bind GFP-Miro1 (Supplementary Figure S9). Additionally, we examined whether the intracellular functional M41-, M85- and M114-Cb could recognize the previously identified domains of Miro1 within living cells. Thus, we expressed the Miro1 domain deletion constructs described above (Figure 3A) along with the Cb constructs in HeLa cells. Subsequent cellular imaging showed that ectopic expression of the C-terminal GTPase domain of Miro1 resulted in specific relocalization of M41-, M85- and M114-Cb to the mitochondrial network, confirming our results from pulldown domain mapping for these Cbs (Supplementary Figures S10A–S10C). However, when we examined Cb binding to endogenous Miro1, we observed a rather diffuse cellular

distribution of the Cb signal and could not detect characteristic mitochondrial structures as seen with an anti-Miro1 antibody staining of Cb expressing cells (Supplementary Figure S10D).

Visualization of Compound-Induced Mitochondrial Dynamics in Living Cells

Our Cb-based imaging results indicated that the M114-Cb shows a slightly better intracellular binding compared to M41- and M85-Cb. Thus, we continued and investigated the utility of this Cb as an intracellular biosensor to track changes in mitochondrial morphology in living cells. For real-time analysis, U2OS cells transiently co-expressing the M114-Cb or a mito-mKate2 construct and GFP-Miro1 were treated with Sorafenib or DMSO as control. Sorafenib has been shown to induce mitochondrial fragmentation and apoptosis in a time-dependent manner (Zhao et al., 2013; Zhang et al., 2017). By time-lapse imaging, we visualized changes in mitochondrial morphology within single cells over a 2 h period with an



imaging interval of 30 min (Figure 6B, Supplementary Figure S11). Following 30 min of Sorafenib treatment, images revealed the collapse of the mitochondrial network reflected by gradual disappearance of elongated mitochondria. After 90 min, condensed mitochondrial networks were visible in the majority of treated cells. Both, the Sorafenib induced mitochondrial morphological transitions as well as normal shape changes observable in the DMSO control were successfully visualized by the M114-Cb.

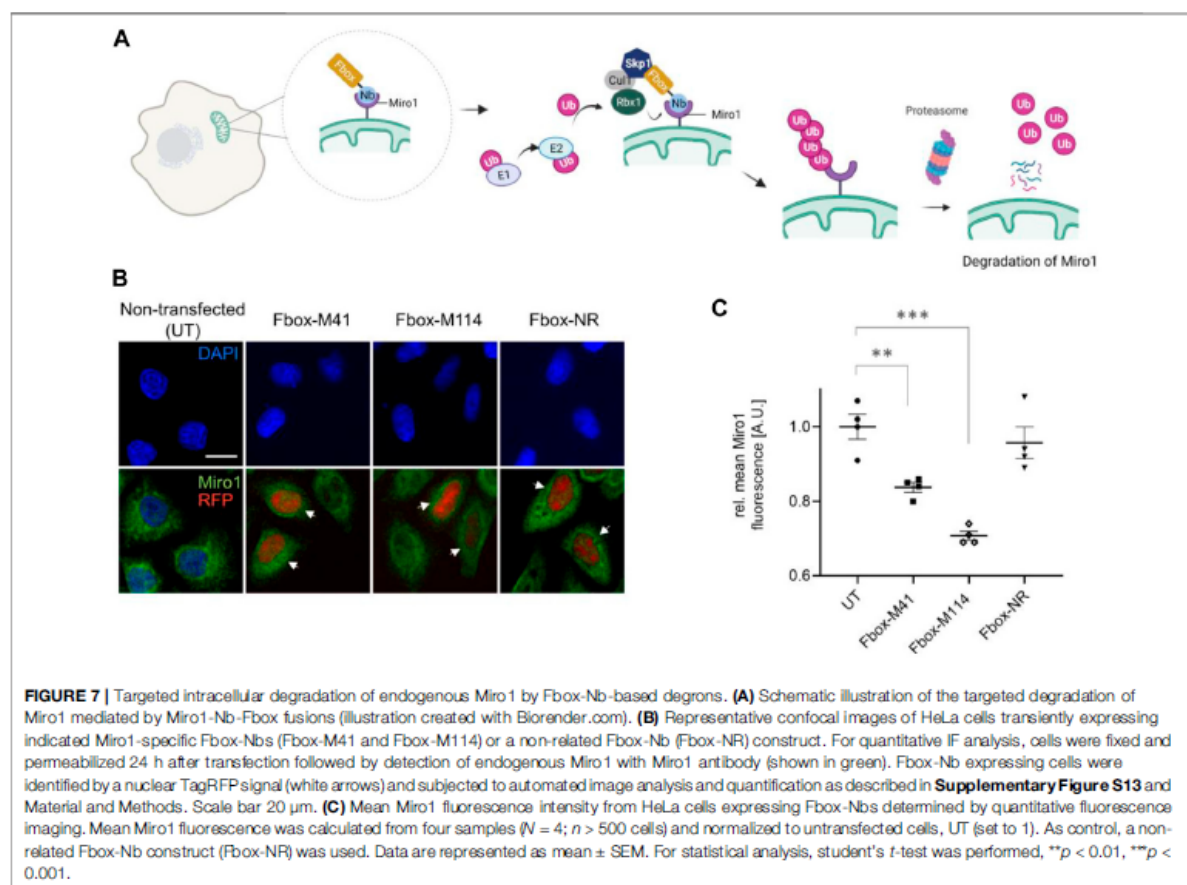
However, although demonstrating that the Miro1-Cbs have the capability to bind and visualize overexpressed, mitochondrial localized Miro1, the experiments shown do not provide an explanation why they failed to visualize endogenous Miro1. Possibly, this is due to a lower intracellular affinity, partial blockade of the detected epitopes by endogenous binding factors or an overall too low cellular expression of Miro1 in the investigated cell types.

Selective Degradation of Miro1 in Living Cells

Previously, it was reported that depletion of Miro1 by pharmacological intervention or genetic silencing rescues mitophagy activation (Hsieh et al., 2019). To test whether our Nbs could also be used to induce targeted degradation of Miro1 in living cells, we engineered genetically encoded Miro1-specific degrons. To this end, we fused the intracellularly functional Nbs, M41 and M114, or the GFP-Nb (GBP) as control, N-terminally to the mammalian Fbox domain to generate a specific loading platform at Miro1 for components of the mammalian E3 ligase complex, namely SKP1 and Cul1 (Figure 7A). Notably,

similar approaches using other Nbs were successfully applied to induce selective protein knockdown within cells or organisms (Causinus et al., 2011; Kuo et al., 2011; Baudisch et al., 2018; Bery et al., 2019; Chen et al., 2021). These Fbox-Nb constructs were cloned into mammalian expression vectors containing an independently transcribed nuclear TagRFP (TagRFP-NLS) to facilitate the identification of transfected cells in cellular imaging analysis. First, we examined whether Fbox-GBP, Fbox-M41, and Fbox-M114 are functional binders and can bind transiently co-expressed GFP-Miro1 in HeLa cells. A clear overlap of the Fbox-Nb signal with GFP-Miro1 after IF staining using an anti-VHH antibody showed that N-terminal fusion of the Fbox domain did not affect intracellular binding of the Nbs (Supplementary Figure S12A). Furthermore, quantification of fluorescence intensity in cells coexpressing the Fbox-Nb constructs and GFP-Miro1 revealed a ~80% reduction of the GFP signal in cells expressing Fbox-M114, ~50% in cells expressing Fbox-M41, and ~50% in cells expressing Fbox-GBP, respectively. Co-expression of a nonspecific Nb-Fbox construct (Fbox-NR) results only in a non-significant reduction in GFP-Miro1 (Supplementary Figures S12B,S12C). To confirm our findings, we further performed immunoblot analysis to validate our findings. Although the degrons show different expression levels we observed a decrease of the GFP-Miro1 in cells expressing the Miro1- or GFP-specific degrons but not the Fbox-NR (Supplementary Figures S12D,S12E).

Although we could not detect a clear relocation of intracellularly expressed Nbs to endogenous Miro1 in the Cb format, we continued and investigated the Fbox fusions with respect to their potential to degrade endogenous Miro1. To that



end, HeLa cells were transfected with Fbox-Nbs, and cellular levels of Miro1 following 24 h of expression were determined by quantitative IF imaging using an anti-Miro1 antibody in cells displaying a nuclear TagRFP signal (**Supplementary Figure S13**). While characteristic mitochondrial structures were still observable (**Figure 7B**), quantification of the antibody signals in a statistically relevant number of cells ($n > 500$) showed a ~16 and ~30% decrease in the IF signal upon expression of Fbox-M41 and Fbox-M114, respectively. Notably, expression of the nonspecific Fbox-NR resulted only in a minor reduction of less than ~4% of the Miro1 signal (**Figure 7C**). From these results we concluded that both specific Fbox-Nb fusion constructs can address endogenous Miro1 in live cells and induce targeted degradation of their antigen.

DISCUSSION

The emerging role of Miro1 in the development and progression of diseases, particularly neurological disorders, underscores the

need for new methods and tools to study this protein in detail at the molecular level (Fatiga et al., 2021; Kassab et al., 2021; Kontou et al., 2021; López-Doménech et al., 2021; Nguyen et al., 2021). Currently, most studies rely on ectopic expression of genetically modified (tagged) Miro1 (Kanfer et al., 2015; Castro et al., 2018; Nemani et al., 2018; Fatiga et al., 2021) which previously has been shown to affect mitochondrial morphology and transport in living cells (Fransson et al., 2006). To expand the ability to study Miro1 in different experimental settings, we developed a collection of Nbs, which were screened for their performance as 1) affinity capture tools, 2) labelling probes for fluorescence imaging, 3) intrabodies for visualization and monitoring of Miro1 in live cells, and 4) as Miro1-specific degrons. In total, we selected seven specific binding molecules, which recognize distinct domains of Miro1 with affinities down to the low nanomolar range. These Nbs can be easily produced in high yields in bacteria and four of them were successfully functionalized by simple chemical conjugation as capture molecules to precipitate Miro1 from soluble cell lysates in the monovalent format.

After determining that the identified Nbs in their monovalent format were not suitable as primary probes for visualization of endogenous Miro1, we decided to convert the most promising candidates, M41 and M114, to a bivalent format. In combination with an advanced labeling strategy using site-specific functionalization *via* sortase tagging in combination with click chemistry, we were able to generate highly functional capture reagents. Our mass spectrometry data indicate that these modified bivalent Miro1 nanotrapers are well suited to capture different isoforms of Miro1 and thus have high potential for future interactome studies. Interestingly, only the site-directed modified bivalent M114-Nb also allowed detection of endogenous Miro1 by immunofluorescence imaging. Consistent with previous findings, this confirms the critical impact of Nb formatting and functionalization for the generation of efficient binding molecules that enable one-step detection of their antigens (Pleiner et al., 2018; Virant et al., 2018; Götzke et al., 2019). A directly labelled bivalent nanobody can bring the fluorophore closer to the target structure compared to conventional staining with a secondary antibody. We previously developed and used such Nb-based imaging probes for the detection of endogenous vimentin using stochastic optical reconstruction microscopy (STORM) (Traenkle and Rothbauer, 2017; Virant et al., 2018). We anticipate that the bi-M114 can be similarly adapted for such advanced imaging techniques on more relevant cells including primary neurons or neurons derived from induced pluripotent stem cells of PD patients.

Besides their application as recombinant capturing and detection tools, numerous studies reported how Nbs can be functionally expressed in the reducing milieu of living cells to visualize subcellular antigen location or to modulate their target structure and function (Rothbauer et al., 2006; Kirchhofer et al., 2010; Maier et al., 2015; Keller et al., 2018; Wagner and Rothbauer, 2020). Accordingly, with M41, M85 and M114, we identified three Nbs, which we converted into intracellularly functional Cbs to visualize Miro1 in living cells. Although, we were not able to detect endogenous Miro1 with Cbs probably due to low levels and a disperse localization of different endogenous isoforms of Miro1, these Cbs could visualize dynamic changes of exogenous Miro1 in time-lapse imaging series. From our treatment studies with Sorafenib we concluded that the Cbs are unaffected by changes in the mitochondrial membrane potential and stably bind their antigen after fixation. This might be advantageous compared to other fluorescent dyes, e.g., the MitoTracker series, which bind to thiol groups within mitochondria and have been shown to be sensitive to changes in the membrane potential (Chazotte, 2011).

To mimic Parkin-mediated Miro1 degradation in living cells, we decided for the application of an artificial nanobody-coupled ubiquitin ligase system. Therefore, we used the M41-Nb and M114-Nb as specific substrate recognition modules for the SKP1-Cul1-Fbox complex to mediate the selective degradation of endogenous and exogenous Miro1. It has to be noted that expression of these degrons did not result in complete depletion of endogenous Miro1. It is possible that the efficacy is due to insufficient binding of endogenous Miro1 yet, which

could be further investigated, e.g., by FRAP (fluorescent recovery after photobleaching) experiments (Maier et al., 2015; Panza et al., 2015). Alternatively, it can be speculated whether other known degron entities, such as fusion with TRIM21 (Clift et al., 2017) or the auxin-dependent degradation system (Daniel et al., 2018), might be better suited for depletion of Miro1 using the target-specific Nbs identified here. Although expression of the degron did not completely deplete endogenous Miro1, the effect was replicable and not observed in the absence of the Fbox protein or in the presence of an unrelated Nb-based degron. For functional studies, CRISPR and RNAi approaches are currently employed to knock down endogenous Miro1 in loss-of-function studies. However, apart from the inherent limitations of each approach, both methods lead to complete loss of Miro1 disrupting mitochondrial homeostasis (López-Doménech et al., 2016; López-Doménech et al., 2021). For this reason, the Nb-based degrons holds great benefit as an option for targeted Miro1 depletion. With the ongoing development of an inducible system applied in PD neuronal models, Fbox-Nb-mediated Miro1 degradation could expand the possibilities to study PD-related mitophagy impairment.

In summary, this study introduces for the first time, an adaptable and flexible toolset of specific Nbs for the multifaceted study of the mitochondrial GTPase, Miro1. In the nanotrap format, the identified Nbs are promising capture tools for the proteomic characterization of Miro1 domain-dependent interactions. Conjugated to fluorophores, Nbs have a distinct potential to be applied as labelling probes for one step detection, e.g., in high-resolution imaging of Miro1 at mitochondria. Formatted into Cbs, formidable intracellular biosensors of Miro1 dynamics within live cells were generated. As intracellular Miro1 binding agents, the Nbs can be easily fused with different protein domains for the targeted modulation of Miro1 in live cells. The versatile applicability of our Nb set underscores its substantial potential as Miro1-specific research toolkit, and we propose that its scope will soon expand to decipher novel and/or further confirm proposed functions of Miro1 in pathophysiological relevant states.

MATERIALS AND METHODS

Nanobody Library Construction

Alpaca immunization with purified Miro1 and Nb library construction were carried out as previously described (Rothbauer et al., 2006). Animal immunization was approved by the government of Upper Bavaria (Permit number: 55.2-1-54-2532.0-80-14). In brief, one alpaca (*Vicugna pacos*) was immunized with recombinant human Miro1 expressed in *E. coli*. After an initial priming dose of 1 mg, the animal received booster injections of 0.5 mg after the third, fourth, seventh and 12th week. 20 ml serum samples collected after the 9th and 13th week were analysed for seroconversion. 13 weeks after the initial immunization, 100 ml of blood was collected and lymphocytes were isolated by Ficoll gradient centrifugation using the Lymphocyte Separation Medium (PAA Laboratories GmbH). Total RNA was extracted using TRIzol (Life Technologies) and

mRNA was reverse transcribed to cDNA using a First-Strand cDNA Synthesis Kit (GE Healthcare). The Nb repertoire was isolated in 3 nested PCR reactions using following primer combinations: 1) *CALL001* and *CALL002*, 2) forward primer set *FR1-1*, *FR1-2*, *FR1-3*, *FR1-4*, and reverse primer *CALL002*, and 3) forward primer *FR1-ext1* and *FR1-ext2* and reverse primer set *FR4-1*, *FR4-2*, *FR4-3*, *FR4-4*, *FR4-5*, and *FR4-6* introducing *SfiI* and *NotI* restriction sites. The sequences of all primers used in this study are shown in **Supplementary Table S1**. The Nb library was subcloned into the *SfiI/NotI* sites of the pHEN4 phagemid vector (Arbabi Ghahroudi et al., 1997).

Nanobody Screening

For the selection of Miro1-specific Nbs, two and three consecutive phage enrichment rounds were performed either with recombinant Miro1 immobilized on Nunc™ Immuno™ MaxiSorp™ tubes (Thermo Scientific) or HEK293-expressed GFP-Miro1 immobilized on ChromoTek GFP-Trap® Multiwell plate (Proteintech). To generate Nb-presenting phages, *E. coli* TG1 cells comprising the Miro1-Nb library in pHEN4 vector were infected with the M13K07 helper phage. 1×10^{11} phages, prepared from culture supernatant by PEG precipitation, were used for each panning process. Extensive blocking of antigen and phages was performed in each selection round with 5% milk or BSA in PBST (PBS, 0.05% Tween 20, pH 7.4) (Pardon et al., 2014).

For the selection process using recombinant Miro1, phages were first applied on immunotubes coated with GFP (10 µg/ml) to deplete non-specific binders and then transferred to immunotubes either coated with Miro1 (10 µg/ml) or GFP (10 µg/ml) as non-related antigen. Incubation steps were performed at RT for 2 h. Washing stringency was increased for each selection round. Bound phages were eluted in 100 mM triethylamine (pH 12.0), followed by immediate neutralization with 1 M Tris/HCl pH 7.4. For the panning process using GFP-Miro1, 2×10^7 HEK293 cells transiently expressing GFP-Miro1 or GFP were harvested and lysed as previously described (Traenkle et al., 2020). GFP-Miro1 and GFP were immobilized respectively on GFP-Trap® Multiwell plates according to manufacturer's protocol. To deplete GFP-specific binding molecules, phages were first applied into wells displaying GFP and then transferred into wells with immobilized GFP-Miro1. All incubation steps of three consecutive selection rounds were performed at 4°C for 2 h under constant mixing. Washing and elution steps were carried out equally as described above. After each panning round exponentially growing *E. coli* TG1 cells were infected with eluted phages and spread on selection plates to rescue phagemids. Antigen-specific enrichment for each round was monitored by counting colony forming unit (CFUs). Following panning, 100 individual clones from both panning strategies were screened by phage ELISA procedures using immobilized Miro1 or GFP-Miro1 as antigen and GFP as control. Bound phages were detected using a horseradish peroxidase-labelled anti-M13 monoclonal antibody (GE Healthcare) and Thermo Scientific™ 1-Step™ Ultra TMB solution.

Expression Plasmids

For the bacterial expression, Nb sequences were cloned into the pHEN6C vector (Rothbauer et al., 2008), thereby adding a

C-terminal sortase tag (LPETG) followed by a His₆ tag for IMAC purification as described previously (Virant et al., 2018). For mammalian expression, the coding sequence for *bivM41* was produced by gene synthesis (Thermo Fisher Scientific) and cloned into the pCDNA3.4 expression vector downstream of an N-terminal signal peptide (MGWTLVFLFLSVTAGVHS) for secretion using restriction enzymes *XbaI* and *AgeI* sites. *bivM114* was generated by insertion of two coding sequences of M114 into the pCDNA3.4 expression vector in three steps: first, M114 sequence was amplified in two separate PCRs using primer sets *bivM114GA-for*, *nterm1273_rev* and *bivM114GA2-for*, *downEcoRI_rev*; second, both amplicons were then fused by overlap-extension PCR with additional use of primers *bivM114FPCR-for* and *bivM114FPCR_rev*; third, the resulting sequence was cloned into *Esp3I*- and *EcoRI*-digested pCDNA3.4 expression vector by Gibson assembly according to the manufacturer's protocol. To generate Miro1-Cbs, Nb sequences were genetically fused with TagRFP by ligation into *BglII*- and *BstEII*-sites of the plasmid previously described as PCNA-chromobody (Panza et al., 2015). The expression construct for GFP-Miro1 was generated by Gibson assembly cloning (Gibson et al., 2009) of three fragments: the pEGFP-N1 vector (Takara Bio) backbone, amplified with the primer set *vectorGA-for* and *vectorGA_rev* and the cDNA of human Miro1 isoform 1 (UniProtKB Q8IXI2-1), amplified in two fragments from the Miro1-V5-HisA plasmid, a gift from Julia Fitzgerald (Grossmann et al., 2019) using the primer set *Miro1fragA-for* and *Miro1fragA_rev* and primer set *Miro1fragB-for* and *Miro1fragB_rev* respectively. Miro1 domain deletion constructs for mammalian expression were cloned from the GFP-Miro1 expression plasmid generated in this study. Respective domains and vector backbone were amplified by PCR using the following primer sets: *nGTP-for* and *nGTP_rev* for eGFP-nGTPase-TMD; *nGTP-for* and *nGTPEF2_rev* for eGFP-nGTPase-EF1-EF2-TMD, *ΔnGTP-for* and *ΔnGTP_rev* for eGFP-EF1-EF2-TMD. Amplicons comprising additional terminal *KpnI* recognition sites were purified, digested with *KpnI/DpnI* and intramolecular re-ligated according to standard protocols. The eGFP-cGTPase-TMD expression plasmid was generated by site directed mutagenesis with the primers, *cGTP-for* and *cGTP_rev* using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer's protocol. The mammalian expression construct for GFP was previously described (Braun et al., 2016). For molecular cloning of the mammalian expression vector pcDNA3_Fbox-Nb-IRES-tRFP-NLS, DNA assembly of the following three fragments was performed. Fragment 1—the complete sequence of plasmid pcDNA3_NSLmb-vhhGFP4, a gift from Markus Amfelder (Addgene plasmid #35579) (Causinus et al., 2011) amplified by PCR using primers *NM95_for* and *NM95_rev*; fragment 2— the IRES sequence, amplified by PCR from a pcDNA3.1 vector variant, pcDNA3.1 (+)IRES-GFP, a gift from Kathleen L Collins (Addgene plasmid #51406) with primers *frag2IRES_for* and *frag2IRES_rev* and fragment 3, generated in two steps. First, an NLS sequence was inserted downstream of the TagRFP sequence in the Cb expression vector described above with

primers *nls-insert_for* and *nls-insert_rev* using the Q5 Site-Directed Mutagenesis Kit (NEB) and the resulting plasmid was used as a template to subsequently amplify the TagRFP-NLS sequence using the primers *frag3-tRFP-nls_for* and *frag3-tRFP-nls_rev*. Fragment assembly was carried out using NEBuilder HiFi DNA assembly Master Mix (New England Biolabs) according to manufacturer's protocol. In the resulting pcDNA3_Fbox-Nb-IRES-tRFP-NLS plasmid the GFP-Nb (vhhGFP4) was replaced by Miro1-Nbs using *BamHI* and *BstEII* restriction sites. All generated expression constructs were sequence analyzed after cloning. For fluorescent labeling of mitochondria in living cells, the mammalian expression vector pmKate2-mito (Evrogen plasmid #FP187) was used.

Protein Expression and Purification

Miro1-Nbs were expressed and purified as previously described (Maier et al., 2015; Wagner and Rothbauer, 2021). Bivalent Nbs were expressed using the ExpiCHO™ system (Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's protocol. For quality control, all purified proteins were analyzed by SDS-PAGE according to standard procedures. Protein samples were denatured (5 min, 95°C) in 2x SDS-sample buffer containing 60 mM Tris/HCl, pH 6.8; 2% (w/v) SDS; 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.02% bromophenol blue prior to analysis. All proteins were visualized by InstantBlue Coomassie (Expedeon) staining. For immunoblotting, proteins were transferred to nitrocellulose membrane (GE Healthcare) and detection was performed using anti-His primary antibody (Penta-His Antibody, #34660, Qiagen) followed by donkey-anti-mouse secondary antibody labelled with AlexaFluor647 (Invitrogen). A Typhoon Trio scanner (GE-Healthcare, excitation 633 nm, emission filter settings 670 nm BP 30) was used for the readout of fluorescence signals.

Affinity Measurements by Biolayer Interferometry

Analysis of binding kinetics of Miro1-specific Nbs was performed using the Octet RED96e system (Sartorius) according to the manufacturer's recommendations. In brief, 2–10 µg/ml solution of biotinylated Miro1-Nbs diluted in Octet buffer (HEPES, 0.1% BSA) was used for 40 s to immobilize the Nb on streptavidin coated biosensor tips (SA, Sartorius). In the association step, a dilution series of Miro1 ranging from 3.9 nM to 1 µM were reacted for 300 s followed by dissociation in Octet buffer for 720 s. Every run was normalized to a reference run using Octet buffer for association. Data were analyzed using the Octet Data Analysis HT 12.0 software applying the 1:1 ligand-binding model and global fitting.

Cell Culture, Transfections and Compound Treatment

HEK293 and U2OS cells were obtained from ATCC (CRL3216, HTB-96), HeLa Kyoto cell line (Cellosaurus no. CVCL_1922) was obtained from S. Narumiya (Kyoto University, Japan) and HAP1 cells were obtained from Horizon Discovery (United Kingdom, catalog number C631). The cell lines were tested for mycoplasma using the PCR

mycoplasma kit Venor GeM Classic (Minerva Biolabs, Berlin, Germany) and the Taq DNA polymerase (Minerva Biolabs, Berlin, Germany). Since this study does not include cell line-specific analysis, cell lines were used without additional authentication. Cell lines were cultured according to standard protocols. Briefly, growth media containing DMEM (high glucose, pyruvate, with GlutaMAX™, Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 10% (v/v) fetal calf serum (FCS, Thermo Fisher Scientific, Waltham, MA, United States) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific) was used for cultivation. Cells were routinely passaged using 0.05% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, United States) and were cultivated at 37°C in a humidified chamber with a 5% CO₂ atmosphere. Transient transfection of U2OS and HeLa Kyoto cells with Lipofectamine 2000 (Thermo Fisher Scientific) was carried out according to manufacturer's instruction. HEK293 cells were transfected with Polyethyleneimine (PEI, Sigma Aldrich, St. Louis, MI, United States) as previously described (Zolghadr et al., 2008; Braun et al., 2016). Compound treatment was done with 10 µM Sorafenib tosylate (Sellekchem) for up to 2 h.

Nanobody Immobilization on NHS-Sephacrose Matrix

2 ml of purified Miro1-Nbs at 1 mg/ml concentration in phosphate-buffered saline (PBS) were immobilized on 1 ml NHS-Sephacrose (GE-Healthcare) according to the manufacturer's protocol.

Sortase Labelling of Nanobodies

Sortase A pentamutant (eSrtA) in pET29 expression vector, a gift from David Liu (Addgene plasmid # 75,144) was expressed and purified as described (Chen et al., 2011). The substrate peptide H-Gly-Gly-Gly-propyl-azide (sortase substrate) was custom synthesized by Intavis AG. For sortase labelling, 50 µM Nb, 250 µM sortase substrate peptide dissolved in sortase buffer (50 mM Tris, pH 7.5, and 150 mM NaCl) and 10 µM sortase were mixed in coupling buffer (sortase buffer with 10 mM CaCl₂) and incubated for 4 h at 4°C. Uncoupled Nb and sortase were depleted by IMAC. Unbound excess of unreacted peptide was removed using Zeba Spin Desalting Columns (ThermoFisher Scientific, Waltham, MA, United States). Azide-coupled Nbs were then labelled by SPAAC (strain-promoted azide-alkyne cycloaddition) click chemistry reaction with 2-fold molar excess of Alexa-Fluor conjugated dibenzocyclooctyne (DBCO-AF647, Jena Bioscience) for 2 h at 25°C. For generation of the bivalent nanotraps, 1 mg of azide coupled bivalent Nb was incubated with 0.5 ml DBCO-Agarose (Jena Bioscience) slurry for 4 h at 25°C. The beads were centrifuged at 2700 × g for 2 min and the supernatant was removed. Samples of the input and flow-through fractions were analysed by SDS-PAGE according to standard protocol. The Nb-coupled beads were washed thrice with 2.5 ml PBS and stored in 1 ml PBS.

Mammalian Cell Lysis and Protein Extraction

3 × 10⁶ HeLa, U2OS, HEK293 and HAP1 cells were each seeded in 100 mm culture dishes (Corning) and cultivated for 24 h. For the

analysis of endogenous Miro1 levels in different cell lines, the cells were harvested after 24 h. Subsequently, cells were washed in PBS (pH 7.4) and harvested, snap-frozen in liquid nitrogen and thawed on ice. Cell pellets were resuspended in 200 μ L of RIPA lysis buffer (10 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate) or 200 μ L lysis buffer [50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml DNaseI, 2.5 mM MgCl₂, 1 $\times$ protease inhibitor cocktail (Serva)] containing either 0.5% NP40 or 1% Triton X-100. Homogenization was achieved by passing the lysate through needles of decreasing gauge and intermittent vortexing for 60 min on ice. Lysates were centrifuged at 18,000 $\times$ g for 15 min at 4°C. Protein concentrations of the soluble fractions were determined using Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific) according to manufacturer's protocol.

Immunoprecipitation

3 $\times$ 10⁶ HEK 293 cells were seeded in 100 mm culture dishes (Corning) and cultivated for 24 h. For the pulldown of endogenous Miro1, the cells were harvested and lysed after 24 h. For the pulldown of GFP-Miro1 or GFP, the cells were subjected to plasmid DNA transfection with equal amounts of expression vectors and cultivated for 24 h. Subsequently, cells were washed in PBS (pH 7.4) and harvested, snap-frozen in liquid nitrogen, stored at -20°C or thawed for immediate use. Cell pellets were homogenized in 200 μ L lysis buffer [50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 μ g/ml DNaseI, 2.5 mM MgCl₂, 1 $\times$ protease inhibitor cocktail (Serva)] by passing through needles of decreasing gauge and intermittent vortexing for 60 min on ice. Lysates were clarified by subsequent centrifugation at 18,000 $\times$ g for 15 min at 4°C. The supernatant was adjusted with dilution buffer (50 mM Tris/HCl, 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF) to 500 μ L. 5 μ L (1%) was added to 2 $\times$ SDS-containing sample buffer (60 mM Tris/HCl, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.02% bromophenol blue; referred to as input). For immunoprecipitation, 40–80 μ L of sepharose-coupled Miro1 Nbs (nanotraps) were added to the protein solution and incubated for 16 h on an end over end rotor at 4°C. As a positive control, 2.5 μ g of rabbit anti-Miro1 antibody (# PA5-42646, ThermoFisher) was added to the protein solution and incubated under the same conditions. For pulldown of immunocomplexes, 40 μ L of an equilibrated mixture of protein A/G-Sepharose (Amersham Biosciences, Amersham, United Kingdom) were added, and incubation continued for 4 h. As a negative control, a non-related Nb (PepNb) immobilized on sepharose beads was used. After centrifugation (2 min, 2700 $\times$ g, 4°C) supernatant was removed and the bead pellet was washed three times in 0.5 ml dilution buffer. On the third wash, the beads were transferred to a pre-cooled 1.5 ml tube (Eppendorf), resuspended in 2 $\times$ SDS-containing sample buffer and boiled for 10 min at 95°C. Samples (1% input, 20% bound) were analysed by SDS-PAGE followed by Western blotting according to standard procedures. Immunoblots were probed with the following primary antibodies: anti-Miro1 (clone 4H4, Sigma-Aldrich), anti-GFP (clone 3H9, ChromoTek), anti-

GAPDH (Santa Cruz) antibody as a negative control to detect unspecific binding to the nanotraps or anti-Vimentin antibody (clone V9, Sigma-Aldrich) for normalization. Immunoblots were scanned on the Typhoon-Trio laser scanner (GE Healthcare) as described and quantitatively analysed using Image Quant TL 8.1 (GE Healthcare). Full scans of western blots from all immunoprecipitation experiments are included in Supplementary Figure S14.

Immunofluorescence

HeLa cells were seeded at 1 $\times$ 10⁴ per well in μ Clear 96 well plates (Greiner). Next day, the cells were transfected with plasmids coding for GFP-Miro1 or a GFP-tagged non-related protein. 24 h post transfection, the cells were washed twice with PBS and fixed with 4% (w/v) paraformaldehyde (PFA) in PBS for 10 min at RT and blocked with 5% BSA in TBST for 30 min at RT. Incubation with purified Miro1 Nbs or AF647 conjugated Nbs (100–200 nM in 5% BSA in TBST) or rabbit anti-Miro1 antibody (# NBP1-89011, Novus Biologicals) was performed overnight at 4°C. Unbound nanobodies were removed by three washing steps with TBST. Unlabelled Miro1 Nbs were detected by addition of a Cy5-conjugated Goat Anti-Alpaca IgG (Jackson ImmunoResearch) according to manufacturer's guidelines. For Miro1 antibody detection, an AF647 conjugated goat anti-rabbit secondary antibody (Invitrogen) was used. Nuclei were subsequently stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and images were acquired immediately afterwards with an ImageXpress™ Micro Confocal High Content Screening system (Molecular Devices) at 40 $\times$ magnification.

Liquid Chromatography-MS Analysis

Miro1 pull-down samples, by application of the mono- and bivalent M41 and M114 nanotraps, were compared to control nanotraps in three technical replicates. Proteins were purified by SDS- PAGE [4–12% NuPAGE tris gel (Invitrogen)] for 7 min at 200 V. Coomassie stained protein gel pieces were excised and applied to tryptic digestion as described previously (Shevchenko et al., 2006). Samples were measured on an Exploris480 mass spectrometer (Thermo Fisher Scientific) online-coupled to an Easy-nLC 1200 UHPLC (Thermo Fisher Scientific). Peptides were separated on an in-house packed [ReproSil-Pur C18-AQ 1.9 μ m silica beads (Dr Maisch GmbH, Ammerbuch, Germany)], 20 cm analytical HPLC column [75 μ m ID PicoTip fused silica emitter (New Objective, Berks, United Kingdom)]. Peptides were eluted with a 36 min gradient, generated by solvent A (0.1% formic acid) and solvent B (80% Acetonitrile in 0.1% formic acid) at a flow rate of 200 nL/min at 40°C. Nanospray ionization at 2.3 kV together with a capillary temperature of 275°C was applied for peptide ionization. Full MS spectra were acquired at resolution 60k within a scan range of 300–1,750 m/z and tandem MS (MS/MS) spectra were acquired at 15k resolution. Maximum Injection Time Mode and automated control target were set to Auto and Standard respectively for full MS and MS/MS scans. The 20 most intense peptides with multiple charge were selected for MS/MS sequencing by higher-energy collisional dissociation (HCD) with a dynamic exclusion of 30 s. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD031218.

Mass Spectrometry Data Analysis

Raw data files were processed using the MaxQuant software suit (version 2.0.3.0) (Cox and Mann, 2008). Spectra were searched against Uniprot *Homo sapiens* database (released 11.12.2019, 96,817 entries), *Vicugna pacos* specific nanotraps and commonly observed contaminants. Peptide mass tolerance was set to 4.5 ppm for MS and to 20 ppm for MS/MS. Peptide and protein false discovery rate was set to 1%. Methionine oxidation and protein N-terminus acetylation were selected as variable modification, while carbamidomethylation on Cysteine was defined as fixed modification. A maximum of two missed cleavages were accepted for specific trypsin digestion mode. For label-free quantification a minimum number of two ratio count was requested. Intensity based absolute quantification was enabled. Statistical analysis was performed with the Perseus software suit (version 1.6.15.0). First, contaminants, reversed and proteins only identified by site proteins were filtered out. Only proteins present in two out of three replicates of each nanotraps were allowed for downstream significance testing. Significantly enriched proteins were determined by *t*-test as Class A with S0 set to 0.1 and FDR threshold ≤ 0.01 or Class B with S0 set to 0.1 and FDR threshold ≤ 0.05 . Additional graphical visualization was performed in the R environment (version 4.1.1). For Miro1 interactome analysis the top 50 protein interactors were classified based on the direct or indirect interaction with Miro1 and the confidence score derived from STRING database. As class 1 interactors, proteins were assigned that are direct interactors with Miro1, and had a confidence score greater than 0.9. Proteins with a direct interaction with Miro1 and a confidence score smaller than 0.9 were annotated as class 2 interactors. Class 3 interactors were annotated based on indirect Miro1 interaction and a confidence score greater than 0.9.

Microscopy and Time Lapse Imaging

8×10^3 U2OS or 1×10^4 HeLa cells/well were plated in a black µclear 96-well plate (Greiner). 24 h after plating, cells were transiently co-transfected with plasmids coding for GFP-Miro1 and M41-Cb, M85-Cb or M114-Cb. The next day, the medium was replaced by live-cell visualization medium DMEM^{high}-2 (Evrogen) supplemented with 10% FBS and 2 mM L-glutamine. For the time-lapse acquisition, U2OS cells transiently expressing GFP-Miro1 and M114-Cb or mito-mKate2 were either treated with 10 µM Sorafenib tosylate or DMSO in live-cell visualization medium and imaged every 15 min for up to 2 h. Images were acquired under standard conditions with the ImageXpressTM Micro Confocal High Content Screening system (Molecular Devices) at 40x magnification.

Image Segmentation and Analysis

For the targeted Miro1 degradation experiments, 8×10^3 – 1×10^4 wildtype HeLa cells or HeLa cells transiently expressing Fbox-Nb-IRES-TagRFP-NLS constructs were fixed and permeabilized in a black µclear 96-well plate (Greiner). Immunofluorescence staining of Miro1 was performed as previously described and images were acquired with ImageXpressTM Micro Confocal High

Content Screening system (Molecular Devices) at 40x magnification. Image analysis was performed with MetaXpress software (64 bit, 6.2.3.733, Molecular Devices) of a statistically relevant number of cells ($n > 500$ cells) for each construct. Using the Custom Module Editor (version 2.5.13.3) of the MetaXpress software, we established image segmentation algorithms to define regions of interest for fluorescence quantification. Based the parameters of size, shape, and fluorescence intensity above local background for DAPI and TagRFP-stained nuclei and for Miro1-stained cytoplasm, we defined whole-cell areas (Supplementary Figure S13). The average Miro1 fluorescence intensities in whole cells were determined for each image followed by subtraction of background fluorescence. The resulting values from the transfected cells were normalized to the non-transfected control. Standard errors were calculated for three independent replicates and student's *t* test was used for statistical analysis.

Analyses and Statistics

Graph preparation and statistical analysis was performed using the GraphPad Prism Software (Version 9.0.0 or higher).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FF and UR conceived the study and analyzed the data. FF, PK, TW, BT, TF, and GJ performed all cellular and biochemical experiments including imaging studies. NB provided recombinant Miro1. SN and AS immunized the alpaca. KZ and BM performed and analyzed mass spectrometry experiments. FF and UR wrote the manuscript with the help of all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2022.835302/full#supplementary-material>

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1 **A nanobody-based toolset to monitor and modify the mitochondrial GTPase Miro1**

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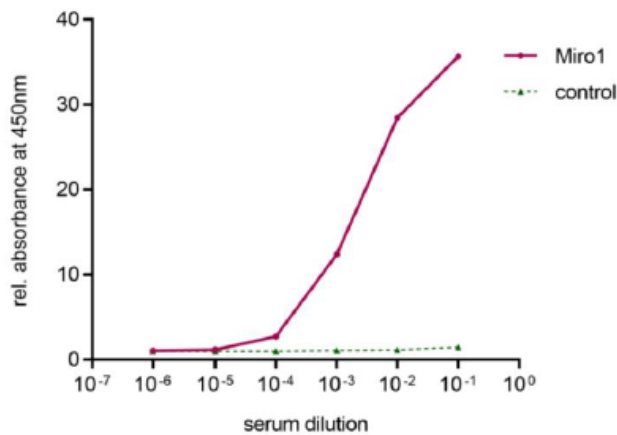
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26 **Supplementary information**

27 **Supplementary Data**

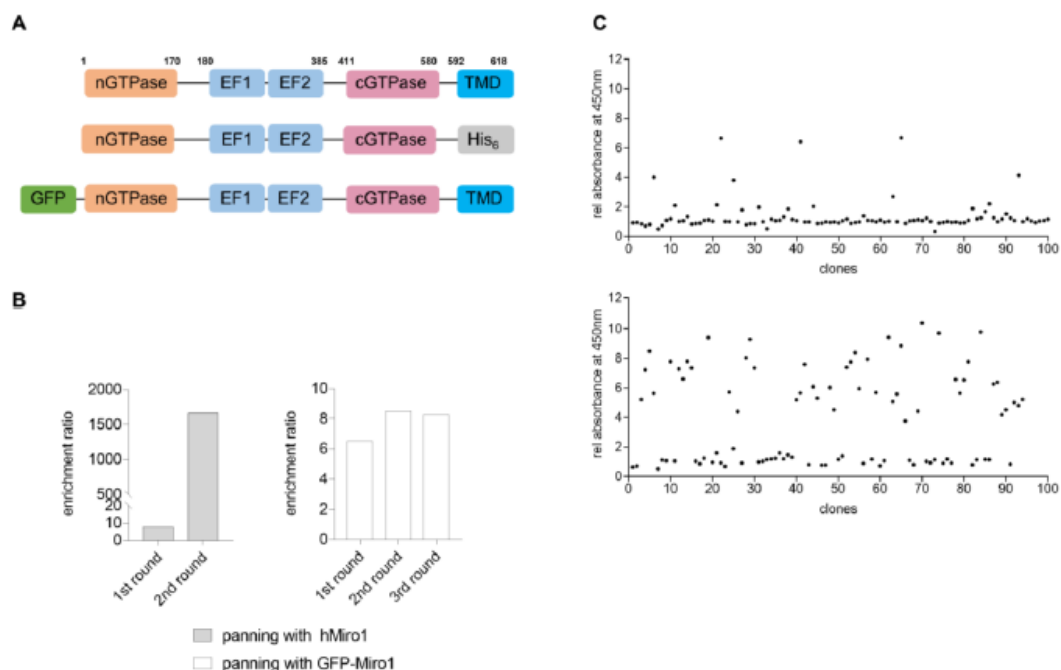


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29 **Supplementary Figure 1. Analysis of seroconversion upon vaccination with Miro1.**

30 To monitor an immune response upon vaccination, a serum sample was taken from the
31 immunized Alpaca (*Vicugna pacos*) on day 63 after starting immunization. Formation of Miro1
32 specific antibodies by the animal was measured in a serum ELISA at indicated dilutions in
33 multiwall plates either coated with hMiro1 or bovine serum albumin (BSA) as negative control.
34 Bound antibodies were detected using an anti-heavy chain antibody and a secondary antibody
35 labelled with horse radish peroxidase. Obtained ELISA signals for hMiro1 were normalized to
36 signals obtained for the negative control.

37



38

39 **Supplementary Figure 2. Enrichment and selection of Miro1 nanobodies (Nbs) by phage**
 40 **display and phage ELISA.**

41 (A) Illustration of wildtype Miro1 and recombinant Miro1 constructs used for phage display.

42 (B) Bar charts showing the enrichment of Miro1-Nb phages after two iterative panning rounds

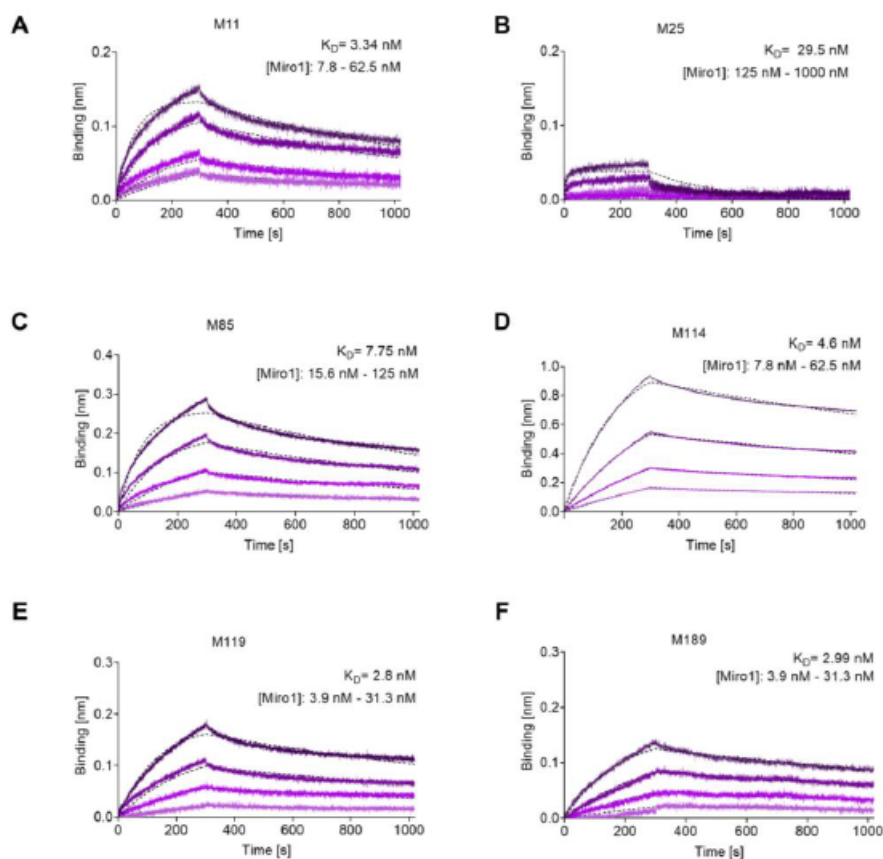
43 against bacterial expressed hMiro1 (left panel, grey bars) and three iterative panning rounds

44 against GFP-Miro1 (right panel, white bars). (C) Phage ELISA profile of 100 eluted phage

45 clones tested for binding to hMiro1 (top panel) or GFP-Miro1 (lower panel). Signal intensities

46 were normalized to a signal obtained for BSA used as negative control.

47

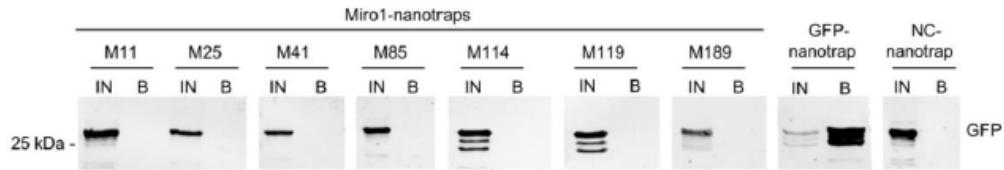


48

49 **Supplementary Figure 3. Affinities of identified Miro1-Nbs.**

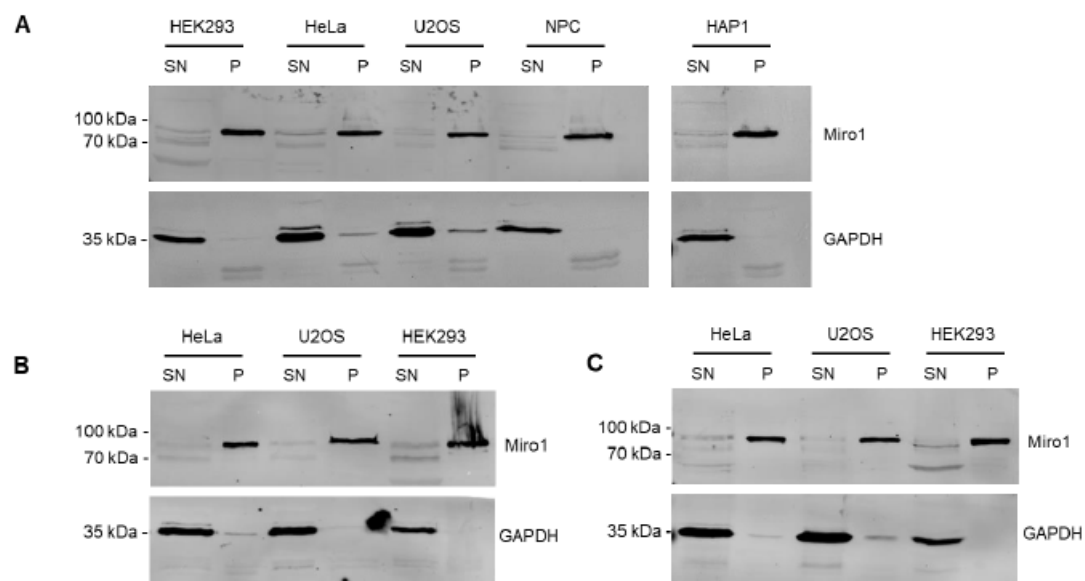
50 Affinities of Miro1-Nbs were analysed by biolayer interferometry based affinity measurements.
 51 Miro1-Nbs were biotinylated and immobilized on streptavidin sensors. Kinetic measurements
 52 were performed by using four concentrations of hMiro1. The sensograms of hMiro1 on M11
 53 (A), M25 (B), M85 (C), M114 (D), M119 (E) and M189 (F) at indicated concentrations
 54 (illustrated with increasingly darker shades from low to high concentration) are shown and
 55 global 1:1 fits are illustrated as dashed lines. A summary of the affinities (K_D), association
 56 constants (K_{ON}) and dissociation constants (K_{OFF}) determined for all seven Miro1-Nbs are
 57 shown in **Figure 1C, Table 1**.

58



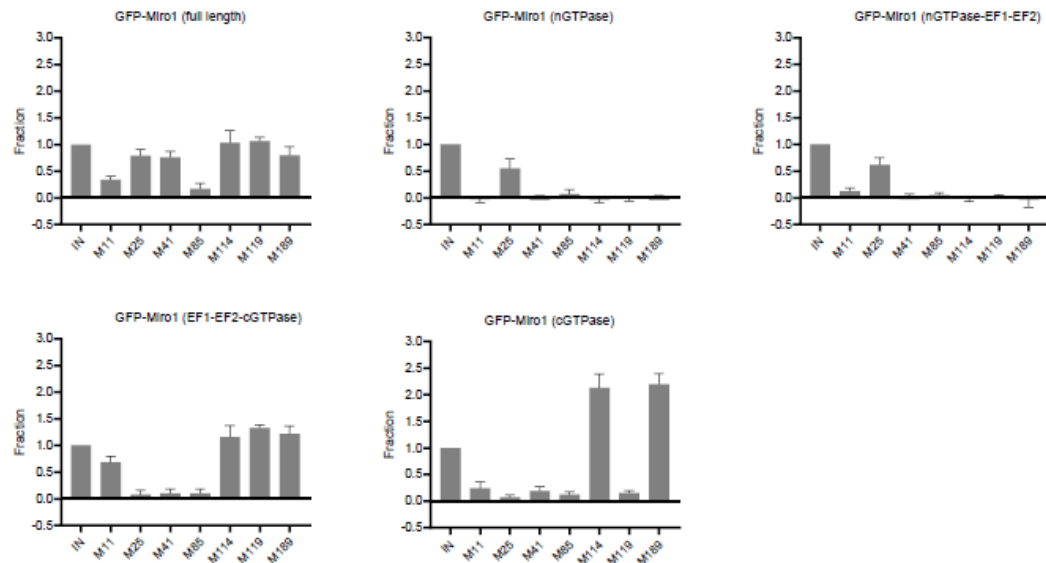
Supplementary Figure 4. Immobilized Miro1-Nbs (nanotraps) do not bind GFP.

Soluble protein fraction of HEK293 cells transiently expressing GFP as control antigen, was adjusted to 2 mg/mL and incubated with equal amounts of nanotraps. Input (IN, 1% of total) and bound (20% of total) fractions were subjected to SDS-PAGE followed by immunoblot analysis using antibodies specific for GFP. As positive control GFP-nanotrap and as negative a non-specific (NC) nanotrap were used.



Supplementary Figure 5. Endogenous Miro1 retains in the insoluble protein fraction in different cell lines.

Representative western blot analysis of 20 µg of soluble (SN) and insoluble/pellet (P) fractions of indicated cells after addition of 0.5% NP-40 (A), or 1% Triton X-100 in the lysis buffer (B), or upon lysis in denaturing radioimmunoprecipitation assay (RIPA) buffer (C). Upper part of the blots were detected with anti-Miro1 antibody. Detection of GAPDH with an anti-GAPDH antibody was used as lysis and loading control.



76

77 **Supplementary Figure 6. Quantification of enriched GFP-Miro1 and Miro1 domains in**
 78 **bound fractions of Miro1 nanotraps**

79 Densitometric evaluation of immunoblots shown in **Figure 3B**. The protein levels detectable
 80 in the bound fractions using an anti-GFP antibody were normalized to the input fraction.

81 Shown are the mean signals from three independent experiments $\pm$ S.D.

82

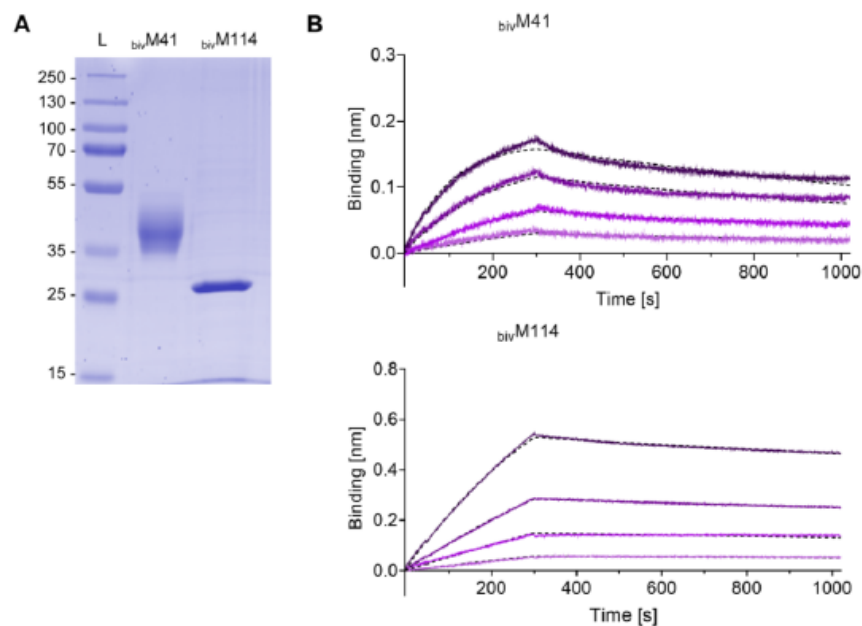


Table 1

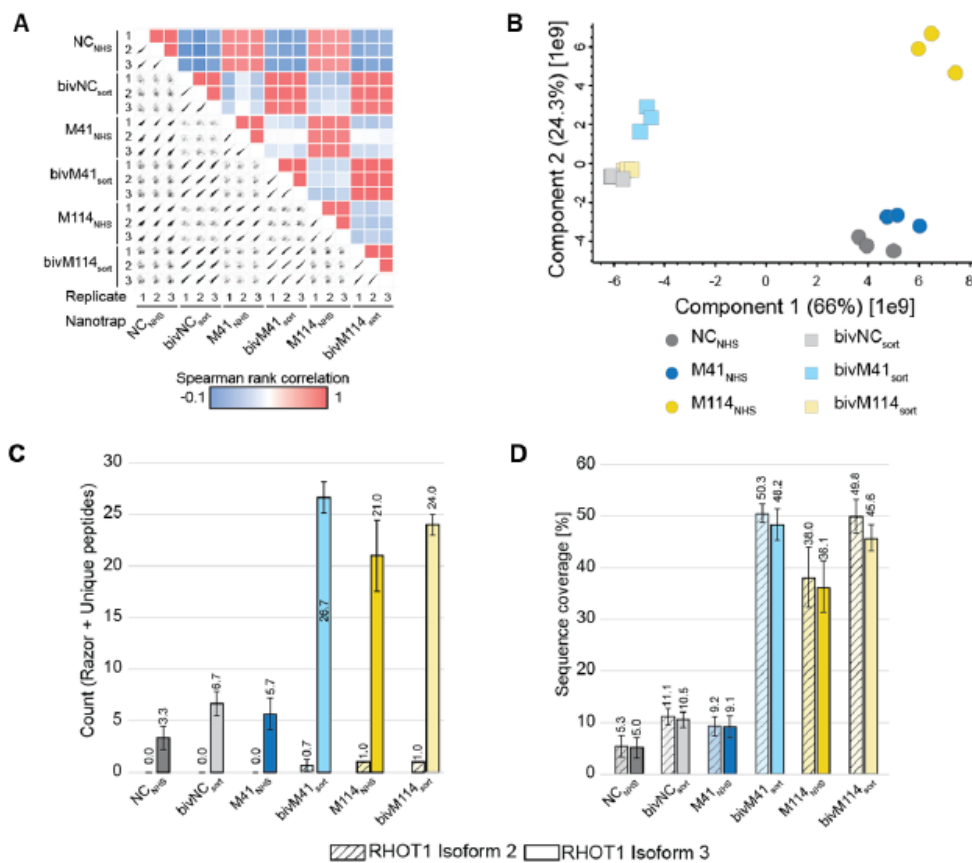
	K_D (nM)	k_{ON} ($10^5 M^{-1} s^{-1}$)	k_{OFF} ($10^{-4} s^{-1}$)	R^2
bivM41	1.86	3.19 ± 0.02	5.95 ± 0.02	0.99
bivM114	2.35	0.78 ± 0.00	1.83 ± 0.01	0.99

83

84 **Supplementary Figure 7. Recombinant expression, purification and characterization of**
85 **bivalent M41- and M114-Nbs.**

86 (A) Coomassie stained SDS-PAGE of 2 μg bivM41- and bivM114-Nbs purified from ExpiCHOTM
87 cells is shown. Differences in running behaviour is due to N-glycosylation of bivM41 upon
88 expression in mammalian (ExpiCHO) cells. (B) Affinities of bivalent Miro1-Nbs were analysed
89 by biolayer interferometry (BLI) based affinity measurements. Bivalent Miro1-Nbs were
90 biotinylated and immobilized on streptavidin sensors. Kinetic measurements were performed
91 by using four concentrations of hMiro1 ranging from 3.9 nM – 31.3 nM (illustrated with
92 increasing concentrations in darker shades). The sensograms of purified Miro1 on bivM41-Nb
93 (top) and bivM114-Nb (bottom) are shown and global 1:1 fits are illustrated as dashed lines.
94 The table summarizes affinities (K_D), association (k_{ON}) and dissociation constants (k_{OFF}), and
95 coefficient of determination (R^2) determined for both bivalent Nbs.

96

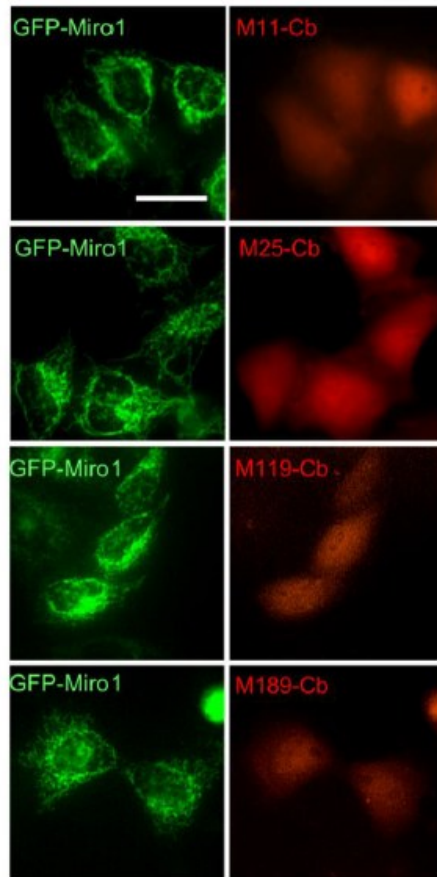


97

98 **Supplementary Figure 8. Enrichment efficiency of bivalent nanotraps.**

99 (A) Multi correlation between replicates and nanotraps. High Spearman rank correlation
 100 between replicates and mono- or bivalent nanotraps. (B) Principle component analysis (PCA)
 101 reflects highest similarity between replicates. 66% of variance between samples explained by
 102 nanotrap valency. (C) Identification of Miro1 (gene name: RHOT1) specific peptides (razor and
 103 unique) for both Miro1 isoforms. (D) Averaged sequence coverage of Miro1 (gene name:
 104 RHOT1) after precipitation with indicated nanotraps. (C, D) Shown are the results from three
 105 technical replicates (n = 3) ± S.D.

106

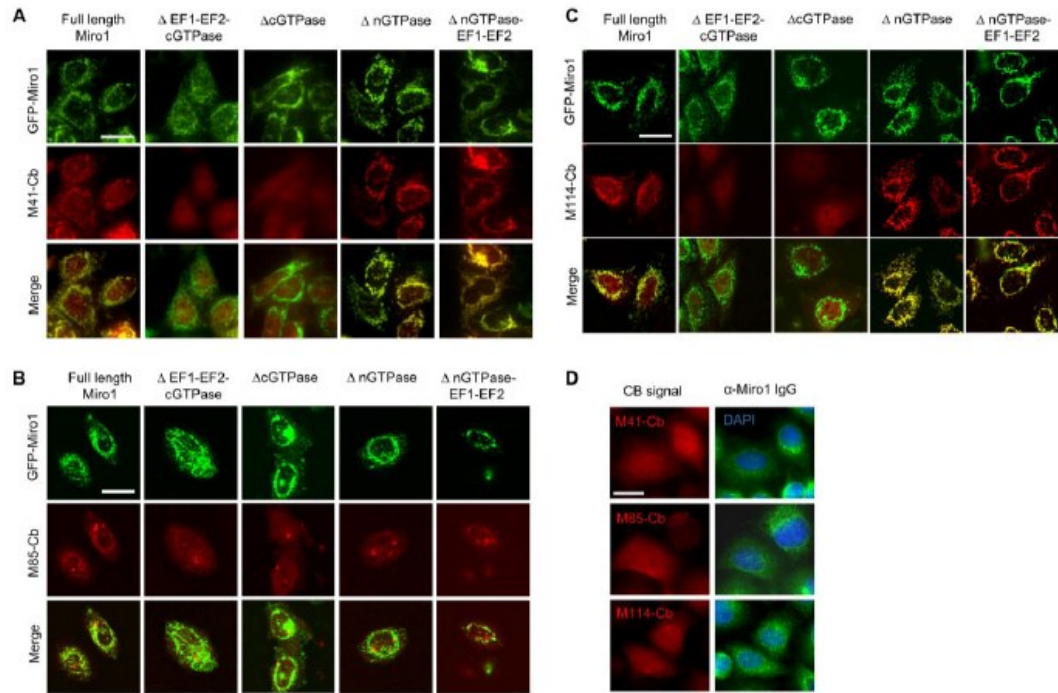


107

108 **Supplementary Figure 9. Detection of intracellular binding capacities of Miro1-Cbs.**

109 Representative fluorescence images of live HeLa cells transiently expressing GFP-Miro1 (left
 110 column) in combination with TagRFP-labelled Miro1-Cbs (right column). Scale bar 20 μ m.

111



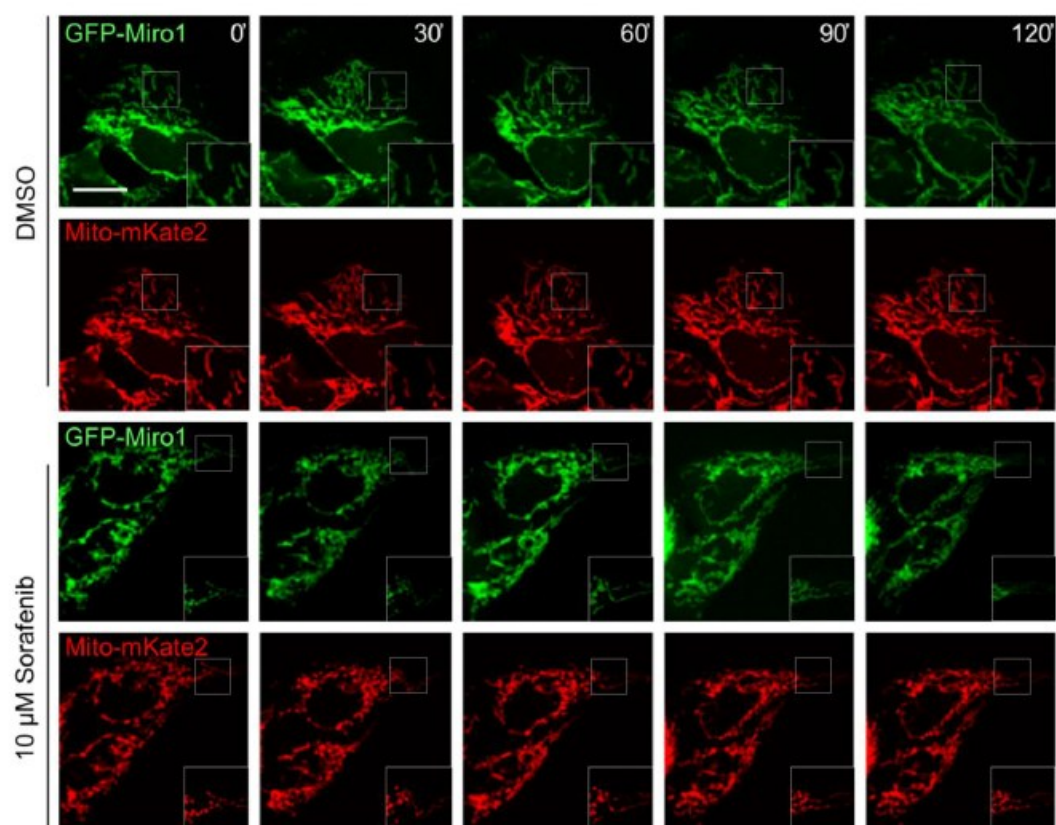
112

113 **Supplementary Figure 10. Intracellular characterization of domain specific binding of**
 114 **selected Miro1-Cbs.**

115 Representative fluorescent images of live HeLa cells transiently expressing GFP-Miro1 and
 116 indicated GFP-tagged Miro1 domain deletion constructs (top row) in combination with
 117 TagRFP-labelled M41-Cb (**A**), M85-Cb (**B**) or M114-Cb (**C**) (middle row). Scale bar 20 μ m.

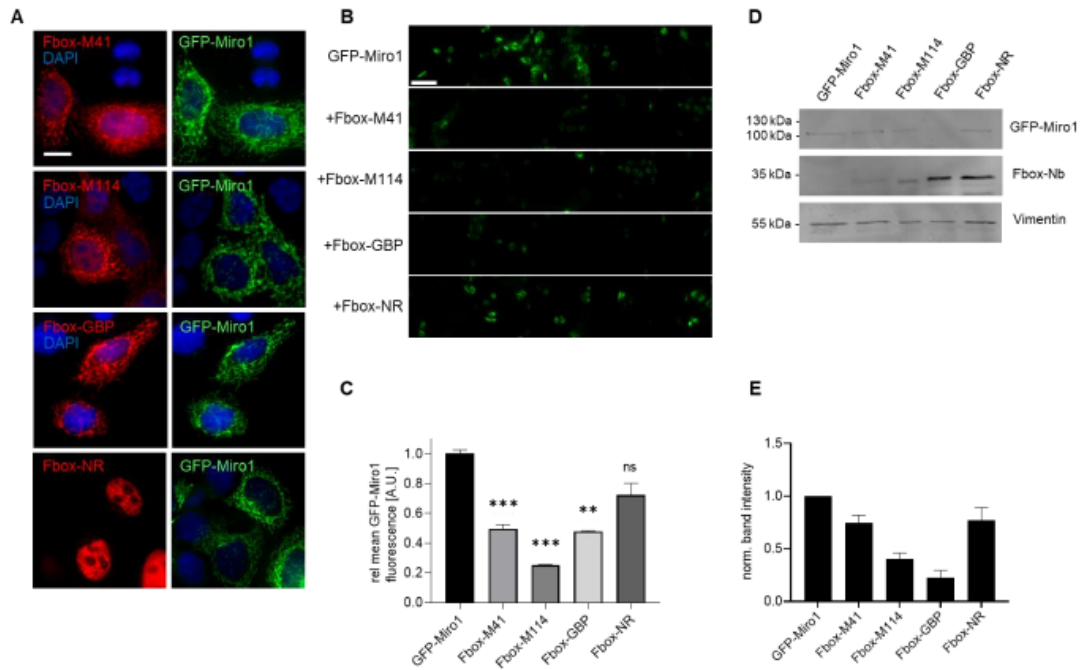
118 (**D**) Immunofluorescence detection of endogenous Miro1 in HeLa cells expressing M41-, M85-
 119 and M114-Cb (left panel) using an anti-Miro1 antibody (right panel). Scale bar 25 μ m.

120



Supplementary Figure 11. Live-cell imaging of mitochondrial phenotypes.

Time-lapse microscopy of U2OS cells transiently expressing GFP-Miro1 and mitoMkate2 (as a mitochondrial marker). To visually track morphological mitochondrial changes, cells were treated with either DMSO as a control (top two rows) or 10 μ M Sorafenib (bottom two rows) followed by time-lapse imaging over a 2 hour period. Shown are representative images of three biological replicates. Scale bar 25 μ m. Squares at the bottom right represent enlargements of the selected image section.



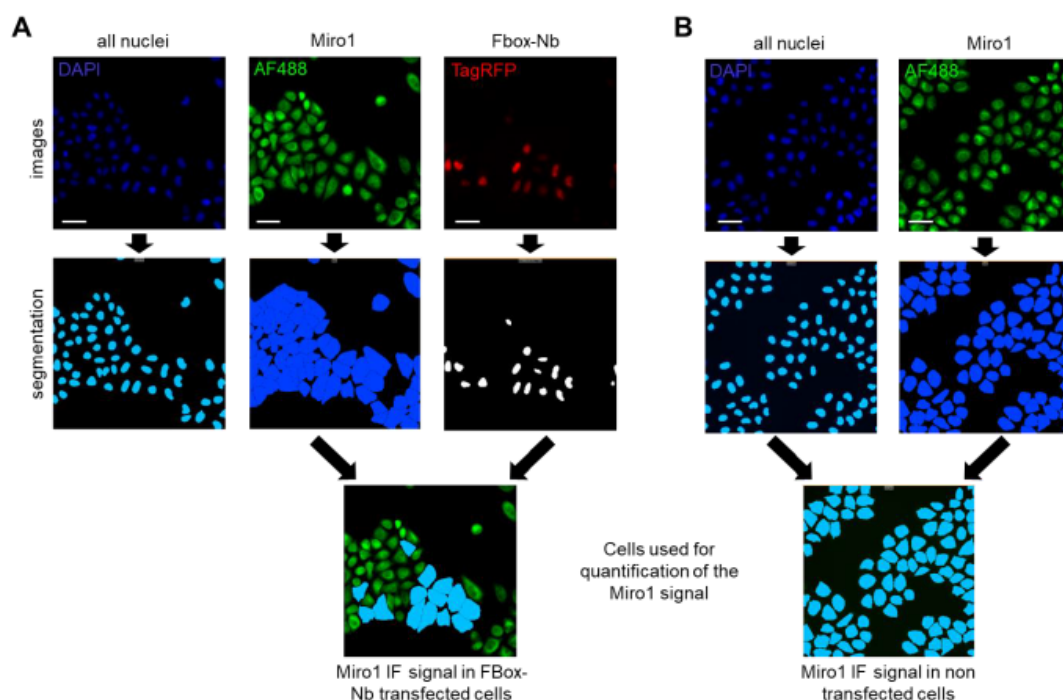
130

131 **Supplementary Figure 12. Targeted degradation of GFP-Miro1 by Fbox-Nb-based**
 132 **degrons in live cells.**

133 (A) Representative confocal images of HeLa cells transiently co-expressing GFP-Miro1 and
 134 indicated Miro1-specific Fbox-Nbs (Fbox-M41, Fbox-M114) or a non-related Fbox-Nb (Fbox-
 135 NR) construct are shown. For quantitative IF analysis, cells were fixed and permeabilized 24 h
 136 after transfection followed by staining with Cy5 conjugated anti-VHH antibody and DAPI. Fbox-
 137 Nb expressing cells were subjected to automated image analysis and quantification as
 138 described in Material and Methods. Scale bar 20 μ m. (B) Representative fluorescence images
 139 of HeLa cells coexpressing GFP-Miro1 and Fbox-Nb constructs. Scale bar 100 μ m. (C) Mean
 140 GFP-Miro1 fluorescence intensity from HeLa cells co-expressing GFP-Miro1 and Fbox-Nbs
 141 constructs were determined by quantitative fluorescence imaging. Fluorescence intensity
 142 values were calculated from three samples (n= 3; >500 cells) and normalized to the GFP-Miro1
 143 signal intensity (set to 1). Positive control; GFP-specific Fbox-Nb (Fbox-NR), negative
 144 control; non-related Fbox-Nb construct (Fbox-NR). Data are represented as mean $\pm$ SEM. For
 145 statistical analysis, student's t-test was performed, **p < 0.01, ***p < 0.001. (D) Representative
 146 immunoblot of lysates of HeLa cells expressing either GFP-Miro1 alone or in combination with

147 the indicated Fbox-Nb constructs. For analysis 10 µg of the soluble fractions were subjected
148 to SDS-PAGE and immunoblotting followed by detection using an anti-GFP antibody (GFP-
149 Miro1) and an anti-VHH antibody (Fbox-Nb constructs). As loading control, endogenous
150 vimentin was detected using an anti-Vimentin antibody. **(E)** Densitometric evaluation of
151 immunoblot analysis. GFP-Miro1 signals from the different cell lysates were normalized to the
152 GFP-Miro1 signal derived from cells expressing GFP-Miro1 only. Shown are the mean signals
153 from three independent experiments $\pm$ S.D.

154

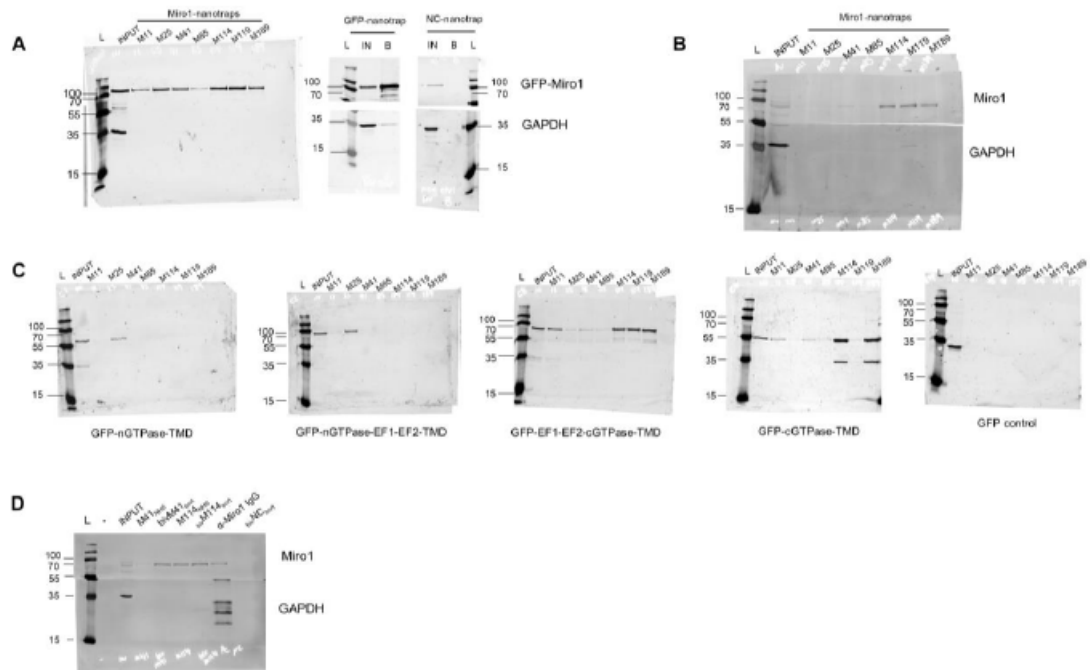


155

156 **Supplementary Figure 13. Workflow showing automated image segmentation used for**
 157 **quantitative immunofluorescence analysis**

158 Representative images of Fbox-Nb transfected (**A**) or untransfected HeLa cell (**B**) fixed and
 159 stained with DAPI and an anti-Miro1 antibody (top row). Image segmentation of stained areas
 160 showing the Miro1 signal and the nuclear TagRFP signal indicating degron (Fbox-Nb)
 161 transfected cells (middle row). Selection of FBox-Nb expressing cells used for quantification of
 162 Miro1 specific antibody signal (bottom row). Scale bar = 50 μ m

163



164

165 **Supplementary Figure 14. Western blot data from immunoprecipitation**

166 (A) Full size of Western blots shown in **Figure 2A**, top halves stained with anti-GFP antibody,
 167 bottom halves stained with anti-GAPDH antibody (B) Full size of Western blots shown in
 168 **Figure 2B**, top half detected by anti-Miro1 antibody, bottom half by anti-GAPDH antibody.
 169 (C) Full size of Western blots shown in **Figure 3B**, detection with anti-GFP antibody. (D) Full
 170 size of Western blots shown in **Figure 5**, top half detected by anti-Miro1 antibody and the
 171 bottom half by anti-GAPDH antibody.

172

173 **Supplementary Table S1.** List of oligonucleotides used in this study

174

Name	Sequence 5' - 3'	purpose
CALL001	GTCCTGGCTGCTCTTCTACAAGG	Nb library generation
CALL002	GGTACGTGCTGTTGAACTGTTCC	
FR1-1	CATGGCNSANGTGCAGCTGGTGGANTCNGGNGG	
FR1-2	CATGGCNSANGTGCAGCTGCAGGANTCNGGNGG	
FR1-3	CATGGCNSANGTGCAGCTGGTGGANAGYGGNGG	
FR1-4	CATGGCNSANGTGCAGCTGCAGGANAGYGGNGG	
FR1-ext1	GTAGGCCAGCCGGCCATGGCNSANGTGCAGCTGGTGG	
FR1-ext2	GTAGGCCAGCCGGCCATGGCNSANGTGCAGCTGCAGGA	
FR4-1	GATGCGGCCGCGNANGANACGGTGACCNGNRYNCC	
FR4-2	GATGCGGCCGCGNANGANACGGTGACCNGNGANCC	
FR4-3	GATGCGGCCGCGNANGANACGGTGACCNGRCTNCC	
FR4-4	GATGCGGCCGCRCTNGANACGGTGACCNGNRYNCC	
FR4-5	GATGCGGCCGCRCTNGANACGGTGACCNGNGANCC	
FR4-6	GATGCGGCCGCRCTNGANACGGTGACCNGRCTNCC	
Miro1fragB_for	CAACAATGGCCATGTATCCACACGTGACACAAGCTGACCTC A	Cloning of GFP-Miro1 mammalian expression construct
Miro1fragB_rev	CAAGCTCTTCAGCAATATCACGG	
Miro1fragA_for	CGTGTGGATACATGGCCATTGTTGTCAATTTAACAAGA	
Miro1fragA_rev	TGGTGGCGGAGGTAGCATGAAGAAAGACGTGCGGATC	
vectorGA_for	TGGCTACCCGTGATATTGCTG	
vectorGA_rev	CGTCTTTCTTCATGCTACCTCCGCCACCACTTC	
nGTP_for	ATTGGTACCTTTTGGCTTCGAGCAAGTTTTGG	Cloning of Miro1 domain deletion constructs
nGTP_rev	ATTGGTACCCTCCTTCTCCTCTGGGC	
nGTPEF2_rev	GCGGGTACCATTTCTTTGAGTTTGTCTTCTGCAGG	
Delta nGTP_for	ATTGGTACCGAGGAGAAGGAGATGAAACCAGC	
Delta nGTP_rev	GCTGGTACCCATGCTACCTCCGCCACC	
cGTP_for	AAAAAACAACTCAAAGAAATGTGTTTCAGATG	
cGTP_rev	CATGCTACCTCCGCCACCACTTC	
bivM114GA_for	GCCGGCGTGCACTCTGAGGTACAGCTGCAGGAGTCGGG	Cloning of bivM114 expression construct
nterm1273_rev	CACCACCGCCAGATCCACCGCCACCTGATCCTCCGCCTCC GCTGCTAACGGTGACC	
bivM114GA2_for	GGTGGATCTGGCGGTGGTGGAAAGTGGTGGCGGAGGTAGT GAGGTACAGCTGCAGGAGTCGGG	
downEcoRI_rev	GTTGTAAAACGACGGCCAGTG	
bivM114FPCR_for	GTCTGTGACCGCCGGCGTGCACTCTGAG	
bivM114FPCR_rev	TTTAATTAAGCGGCCGCGAATTGTTGTAAAACGACGGCCAG TG	
NM95_for	TAATCTAGAGGGCCCTATTCTATAGTG	Cloning of Fbox-M41 , Fbox-M114 and Fbox-NR constructs
NM95_rev	GCTGGAGACGGTGACCTG	
frag2IRES_for	CCCAGGTCACCGTCTCCAGCTAACTAGAGGTTAACGAATTC	
frag2IRES_rev	GGTTGTGGCCATATTATC	
nls-insert_for	AAGAAGAGGAAGGTTTGAGCGGCCGCGACTCTA	
nls-insert_rev	CTTAGGGCTGCCTCCATTAAAGTTTGTGCCCCAGTTTGCTAG	
frag3-tRFP-nls_for	ATGATAATATGGCCACAACCATGGTGTCTAAGGGCGAAG	
frag3-tRFP-nls_rev	GAATAGGGCCCTCTAGATTAAACCTTCCTCTTCTTCTAGG	

175

176 **Supplementary Table S2.** Amino acid sequences of all selected Miro1-Nbs.

Miro1-Nbs	Amino acid sequence
M11	QVQLVESGGGSVQPGGSLRLSCAASGFSFSSNVMSWARQAPGKGLEWVSGIYV DGRTHYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAAGDLSGYSFSP YDYDYWGQGTQVTVSS
M25	HVQLVESGGGLVQPGGSLRLSCIASGFTFSDVGMWYRQIPGKERDMVASITSFG DQTSYAHSVKGRFTISRDNAKNTVYLQMNTLIPDDTAMYYCTTVLGRERRWGQG TPVTVSS
M41	EVQLVESGGGLVHPGGSLRISCAASGFSFNSSAMSWARQAPGKGLEWVSGIQAD GTTGYAHSVKGRFNISRDNAKNIVYLQMSSLKPEDSAVYYCAKEGWSTVVFGRD YWGQGTQVTVSS
M85	DVQLVESGGGKVQSGGSLRLSCAASGSKLDDYSIGWFRQAPGKREGISCITMK TGSFEYVDSVKGRFTISADNAKNTVHLQMNNLKPEDTGIIYCAALRSRGLFCVTSP YEYDLWGQGSPTVTVSS
M114	EVQLQESGGGLVQPGGSLRLSCTASGFPVSSAAMSWARQSPGKELEWVSGIYT DGSADYADSLKGRFTISRDDVENTINLQMNSLKPEDTAVYYCKTDWWAGPSGYV AVWGQGTQVTVSS
M119	DVQLVESGGGLVQPGGSLRLSCATSGFPLDNYAIGWFRQAPGSEREGVSCISS GSYFPGSGSRTHYAHSVKGRFTISRDKAKNTVYLQMDNLKPEDTAVYYCAAVPV PSIRTAETMCVRGSLSEFISWGQGSQVTVSS
M189	EVQLVESGGGLVQPGGSLRLSCTASGFPVSSAAMSWARQSPGKELEWVSGIYTD GSADYADSLKGRFTISRDDVENTINLQMNNLKPEDTAIYYCNANFFEGSWYDYWG QGSPVTVSS

177

178

179 **Supplementary Table S3. MS Dataset of endogenous Miro1 enrichment by Miro1**
180 **monovalent and bivalent nanotraps (.xlsx file)**

181 **(A)** Description of terminologies in the Miro1 nanotraps MS proteomic analysis Dataset **(B)**
182 Protein Groups enriched by bivM41_{sort} and bivM114_{sort} nanotraps **(C)** Protein Groups
183 enriched by monovalent M41_{NHS} and M114_{NHS} nanotraps. **(D)** Enrichment levels of Ras
184 GTPases by bivM41_{sort} and bivM114_{sort} nanotraps. **(E)** Enrichment levels of Ras GTPases by
185 monovalent M41_{NHS} and M114_{NHS} nanotraps.

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Theresa