HDLBP binds ER-targeted mRNAs by multivalent interactions to promote protein synthesis of transmembrane and secreted proteins

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The biological role of RNA-binding proteins in the secretory pathway is not well established. Here, we describe that human HDLBP/Vigilin directly interacts with more than 80% of ER-localized mRNAs. PAR-CLIP analysis reveals that these transcripts represent high affinity HDLBP substrates and are specifically bound in their coding sequences (CDS), in contrast to CDS/3′UTR-bound cytosolic mRNAs. HDLBP crosslinks strongly to long CU-rich motifs, which frequently reside in CDS of ER-localized mRNAs and result in high affinity multivalent interactions. In addition to HDLBP-ncRNA interactome, quantification of HDLBP-proximal proteome confirms association with components of the translational apparatus and the signal recognition particle. Absence of HDLBP results in decreased translation efficiency of HDLBP target mRNAs, impaired protein synthesis and secretion in model cell lines, as well as decreased tumor growth in a lung cancer mouse model. These results highlight a general function for HDLBP in the translation of ER-localized mRNAs and its relevance for tumor progression.

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In eukaryotic cells, the localization of functional protein products is largely determined by the site of their translation. While soluble proteins are translated in the cytosol, cotranslational targeting to the endoplasmic reticulum (ER) enables newly synthesized proteins to enter the secretory pathway, resulting in their secretion or membrane integration. The canonical secretory pathway initiates in the cytosol with the synthesis of the hydrophobic targeting signal (signal peptide or transmembrane domain). Subsequent binding of the signal recognition particle (SRP) to the nascent peptide results in ribosome elongation arrest and formation of the ribosome nascent chain complexes (RNCs). This allows the re-localization of the cytosolic SRP-RNC to the ER membrane via the SRP receptor and translocation of the nascent peptide to the ER lumen. In recent years, a non-canonical SRP-independent pathway was discovered in yeast along with the evidence for the recruitment of the SRP to the mRNA prior to ribosome engagement and SRP-independent ER targeting. This raises the possibility of the existence of yet unknown mechanisms for the recognition of membrane-bound mRNAs.

The potential role of regulatory elements in mRNA sequences for ribosome elongation arrest and nascent chain recognition are poorly understood. Several studies have identified elements within coding sequences (CDS) and 3′ untranslated regions (3′ UTRs) that may distinguish ER-bound from cytosolic mRNAs. However, trans-acting factors that may be responsible for the recognition of such elements are unknown. Recently, it was observed that a small subset of mRNAs that encode soluble proteins may also be localized and translated at the ER, indicating additional mechanisms that regulate the fate of a localized mRNA. While there is evidence for subpools of ER-associated ribosomes that interact with pyruvate kinase in muscle, comprehensive differences in the composition, assembly and active translation states of cytosolic and ER-bound ribosomes have not been identified. Furthermore, a previously unknown variant of the ribosome-dependent nonsense-mediated decay (NMD) pathway was discovered at the ER, hinting at an additional layer of regulation for ER-bound mRNAs. In summary, the translational fate of mRNAs encoding soluble and membrane proteins may be tightly regulated by trans-acting factors such as RNA-binding proteins, which could function beyond the canonical SRP-dependent model.

HDLBP (also known as VIGILIN) is a conserved and ubiquitously expressed RBP localized both to the cytosol and the ER membrane. It contains 15 hnRNPK-homology (KH) RNA-binding domains (RBDs) and KH domains are high-affinity RNA-recognition elements (RREs), most commonly tetranucleotides as discovered in yeast along with the evidence for the recruitment of the SRP to the mRNA prior to ribosome engagement and SRP-independent ER targeting. This raises the possibility of the existence of yet unknown mechanisms for the recognition of membrane-bound mRNAs.

Here, we assayed HDLBP binding sites in a transcriptome-wide manner by PAR-CLIP and discovered their potential function as selective sequence determinants of ER-bound mRNAs. HDLBP directly and specifically interacted with more than 80% of all ER-localized mRNAs and was primarily bound to long CU-rich motifs in their CDS, a unique feature which is much more frequently found in membrane-bound compared to cytosolic mRNAs. Biochemical, transcriptomic, and proteomic methods were used to evaluate the functional consequences of HDLBP absence on ER translational efficiency, protein synthesis, and secretion and highlighted its requirement for these biological processes. Finally, we expanded our findings to an in vivo system and evaluated the effect of the absence of HDLBP on tumor formation capacity.

Results

HDLBP directly interacts with ER-targeted mRNAs. To functionally characterize the interactions of HDLBP with the localized transcriptome, we quantified cytosolic and membrane-bound mRNAs in HEK293 cells. Using a subcellular chemical fractionation approach, we obtained cytosol and membrane fractions, as evidenced by the presence or absence of compartment-specific protein markers (Supplementary Fig. 1a). To obtain localized mRNA profiles, we next performed mRNA-seq from whole-cell, cytosol, and membrane fractions (Supplementary Fig. 1b), resulting in highly reproducible mRNA abundance quantifications (Supplementary Fig. 1c). Due to the bimodal distribution of membrane-to-cytosol enrichment (Supplementary Fig. 1a), we were able to classify over 7000 mRNAs according to their partitioning between the cytosol and membrane (Supplemental Data 1). To validate this result, we compared the enrichment values between mRNAs that encoded or lacked co-translational targeting signals. As expected, we found that mitochondrial DNA-encoded proteins, as well as signal peptide (SP) and transmembrane helices (TM) encoding mRNAs had much higher membrane enrichment when compared to mRNAs encoding post-translationally targeted tail-anchored proteins or nuclear DNA-encoded mitochondrial proteins (Fig. 1b), validating our approach.

Successful quantification of mRNA localization prompted us to determine the whole-cell HDLBP-bound transcriptome in HEK293 cells by PAR-CLIP (Supplementary Fig. 1d, e). The majority of FLAG/HA-HDLBP cross-linking signal, detected as T-C transitions, was present in coding sequences (CDS) and 3′ untranslated regions (3′ UTRs) of mRNAs (Supplementary Data 2, 3), as well as in rRNA and tRNAs (Fig. 1c). After expression-level normalization (Supplementary Fig. 1f, g), the PAR-CLIP T-C signal of membrane-bound mRNAs was normally distributed without mRNA abundance bias (Fig. 1d), suggesting high specificity of HDLBP interactions for this pool of mRNAs. Interestingly, high HDLBP PAR-CLIP enrichment in membrane-localized mRNAs was mostly due to high CDS interactions rather than 3′ UTR binding (Supplementary Fig. 1h). We estimated that more than 80% of the total membrane-bound mRNA pool was bound by HDLBP (Fig. 1e). The unbound membrane-localized mRNAs included 13 mitochondrial DNA-encoded genes (Supplementary Fig. 1i), which were not expected to be HDLBP targets since HDLBP is not localized to mitochondria. Correspondingly, HDLBP-bound mRNAs were highly enriched for SP and TM helix encoding transcripts, while a significantly weaker binding was observed for cytosolic mRNAs and those encoding mitochondrial proteins (Fig. 1f). In addition, we found that mRNAs encoding tail-anchored membrane proteins were not enriched, confirming that HDLBP binds to the endoplasmic reticulum (ER)-targeted mRNAs with high specificity.
Differential HDLBP binding to transcript regions of membrane-bound and cytosolic mRNAs. To determine the molecular characteristics of HDLBP–RNA interactions, we first examined the relative distribution of HDLBP cross-linking positions within mRNA transcript regions. High reproducibility of T-C positional counts was observed (Supplementary Fig. 2a), allowing us to construct high-resolution maps of HDLBP cross-linking profiles. We observed reciprocal binding of HDLBP to CDS and 3′ UTR regions depending on mRNA localization. HDLBP interacted predominantly with the CDS of membrane-bound mRNAs, whereas for cytosolic mRNAs the binding was more prominent in the 3′ UTR (Fig. 2a). To address the relationship between CDS, 3′ UTR binding, and mRNA localization, we calculated the ratio between length-normalized T-C transitions detected in CDS versus 3′ UTR and found that membrane-localized mRNAs had higher ratios compared to cytosolic transcripts (Fig. 2b). In addition, the contribution to the total T-C crosslink signal mostly originated from high CDS binding for membrane-bound mRNAs (Fig. 2c). Comparison between T-C transition patterns in membrane-bound mRNAs IGF2R and COL2A1, and cytosolic HNRNPUL1 and FTL transcripts (Fig. 2d and Supplementary Fig. 2b), supported the conclusions from the transcriptome-wide analysis. In summary, HDLBP target mRNAs are primarily membrane-localized and bound in the CDS, while in cytosolic mRNAs HDLBP binding is on average equal between CDS and 3′ UTR.

RNA-recognition elements for HDLBP binding are more common in membrane-bound mRNAs. We next explored the primary sequence characteristics underlining the specificity of HDLBP for membrane-bound mRNAs. We ranked crosslinked seven-mers by their median crosslink signal per transcript (Supplementary Fig. 3a) and by the frequency of T-C crosslink positions relative to all crosslinked seven-mers (Fig. 3a). These two metrics were compared between CDS and 3′ UTR of mRNAs either localized to the membrane or the cytosol. We found that HDLBP is most frequently crosslinked to CU-containing seven-mers with a variable number of UUC/UC/CUU/CU repeats, located in the CDS of membrane-bound mRNAs (Fig. 3a, b and Supplementary Fig. 3a).
We asked if HDLBP binding is determined by the differential sequence composition of cytosolic and membrane-bound mRNAs. For this purpose, we determined the frequency of all possible seven-mers within whole 3' UTR and CDS sequences and compared it to the frequency of HDLBP crosslinked seven-mers. While the top HDLBP crosslinked seven-mers were not among the most frequent seven-mers in the transcriptome, they showed a significantly higher occurrence (P = 2.7e-07) in the CDS of membrane-bound compared to cytosolic mRNAs (Supplementary Fig. 3b).

Since HDLBP is composed of 15 KH domains,26,33 we next addressed the possibility that it could recognize longer RREs.40 Therefore, the difference in the frequency between membrane-bound and cytosolic mRNAs was calculated for the 40 most highly crosslinked k-mers with a length between 4 and 12 nucleotides. Crosslinked k-mers were generally more frequent in sequences of membrane-bound mRNAs than in cytosolic mRNAs (Fig. 3c). In addition, the greatest difference between membrane-bound and cytosolic mRNAs was observed for the longest k-mers (10–12 nt) (Fig. 3c). Therefore, membrane-bound mRNAs contained a significantly higher number of longer high-affinity RREs for HDLBP binding than cytosolic mRNAs (Fig. 3d). On the other hand, the occurrence of crosslinked k-mers was comparable between CDS and 3' UTR of cytosolic mRNAs (Supplementary Fig. 3c, d), and corresponded to the equal distribution of HDLBP crosslinks between these two transcript regions (Fig. 2c). In summary, the specific binding of HDLBP to the CDS of membrane-bound mRNAs can at least in part be explained by the differential k-mer composition of membrane-bound and cytosolic mRNAs.

High-affinity multivalent HDLBP interactions are more frequently formed in membrane-bound mRNAs. Due to the high number of KH domains, we next reasoned that HDLBP could recognize RREs interspaced with unbound nucleotides, giving rise to multivalent interactions. By counting the frequency of most frequently crosslinked four-mers in 40 nt regions around all detected crosslink positions (Supplementary Fig. 3f), we found that the regions with the highest four-mer frequency also showed the highest crosslink values within these regions (Fig. 3e). Therefore, the highly multivalent HDLBP binding sites also resulted in high-affinity interactions. In addition, the average positional crosslink signal within regions with the highest multivalency revealed that several positions upstream (~13 nt) and downstream (~16 nt and ~20 nt) of the crosslink were specifically crosslinked (Fig. 3f). Therefore, high-affinity HDLBP sites contain 3–4 RNA elements positioned several nucleotides apart giving rise to binding sites of ~40 nt in length. Notably, multivalent interactions resulted in high-affinity interactions both in the CDS and 3' UTR of membrane-bound and cytosolic mRNAs, respectively (Supplementary Fig. 3e).

To explore the potential of membrane-bound and cytosolic mRNAs to form multivalent interactions with HDLBP, we next calculated the occurrence of four-mers within 30-nt sliding windows of mRNA sequences. We observed that the local frequencies of HDLBP crosslinked four-mers were significantly higher in CDS of membrane-bound than in cytosolic mRNAs (Fig. 3g). This effect was absent for a group of unbound four-mers, confirming that membrane-bound CDS contain a high density of local HDLBP recognition elements that give rise to multivalent interactions.

To determine the RNA substrate specificity in more detail, we expressed and purified recombinant full-length HDLBP (FL) and protein variants (A–D) harboring different sets of KH domains (Fig. 3h and Supplementary Fig. 3i). Using a fluorescent anisotropy assay, we determined the apparent dissociation constants (Kd) for the respective protein–RNA substrate combinations, which are summarized in Fig. 3h. The full-length HDLBP bound with high-affinity to regions of TFRC mRNA (TFRC_1 and _2), which were selected based on our PAR-CLIP data. Mutations in one of the TFRC sites (TFRC_1_mut) reduced binding. Using artificial sequences, we could show that HDLBP interacted strongly with CCU- and CUU-oligomers, whereas affinity to a CAA oligomer was about tenfold reduced. In addition, HDLBP, albeit with reduced affinity, is bound to a region in the ES6 region of the 18 S rRNA. Interestingly, HDLBP construct B, comprising KH domains 5 through 9, bound with nearly similar affinities to the tested mRNA and 18 S rRNA sequences as the full-length protein, suggesting that the central part of HDLBP is important for recognition and interaction with different RNA substrates. The 5 C-terminal KH domains HDLBP showed reduced binding to the TFRC mRNA region when compared to full-length HDLBP. For the N-terminal HDLBP...
fragment A (KH0-KH4) we were unable to identify a high-affinity RNA substrate.

To understand the impact of multivalent interactions on HDLBP binding affinity, we next performed the in vitro HDLBP binding assay using RNA molecules with different numbers of bound four-mer motifs within a longer (34 nt) sequence (Fig. 3i). We determined significantly lower Kd values for synthetic RNA molecules containing three HDLBP-bound four-mers (CUUC or UCUU) than those that harbored only 2. Therefore, HDLBP (KH5-9) has ~3–5-fold higher affinity for RNAs with higher multivalent potential. These findings strongly support our conclusions from PAR-CLIP analysis and confirm that HDLBP
binds with high affinity to long RNA regions with multivalent interactions.

HDLBP interacts with the translational apparatus. Since HDLBP showed specific interactions with the CDS of membrane-bound mRNAs, we next explored the possibility that it plays a role in the translational regulation of ER-targeted mRNAs. HDLBP PAR-CLIP showed binding to 18S rRNA with two major binding sites located in expansion segment 68B and helix 16 (Fig. 4a). These sites likely play a regulatory role in translation initiation and elongation41,42 due to their proximity to the binding sites of eukaryotic initiation and elongation factors (EIF4A, EIF4G1, and EEF2) (Supplementary Fig. 4a). Next, we examined HDLBP binding to the 7SL RNA component of the signal recognition particle (SRP), an RNP required for cotranslational targeting of nascent polypeptide-associated complexes to the ER membrane. In 7SL RNA, we observed HDLBP contacts that were distinct from the binding patterns of other RBPs (IRE1, SSB, and MOV10) (Fig. 4b). HDLBP crosslinks were located in helices (5d–f, 6, 8a) of the large (S) domain and at the unpaired uridines of the small Alu region (Supplementary Fig. 4c). The percentage of SRP over total T-C crosslinks was comparable to other known or expected 7SL RNA interactors (IRE1 and SSB) and ~10-fold higher than for MOV10, which does not strongly bind 7SL RNA (Supplementary Fig. 4d). In summary, the HDLBP interacts with the 40S subunit of the ribosome at positions that are also close to the SRP contact sites (Fig. 4b).

In order to ensure that these contacts resulted from stable interactions, we confirmed various HDLBP RNA targets by RNA immunoprecipitation (RIP) experiments (Fig. 4c). Results corresponded to the conclusions of PAR-CLIP experiments with 7SL RNA showing a moderate enrichment, while several membrane-bound mRNAs (YWHAZ, ATP1A1, CD46, and IGF2R) showed high enrichment, whereas an mtDNA-encoded mRNA (MT-CO1) showed no enrichment. These results supported the conclusion that HDLBP forms stable interactions with RNA species identified by PAR-CLIP.

To validate the interaction with the translational apparatus by an orthogonal method, we next profiled HDLBP-proximal proteins using BioID43, a proximity labeling assay in HEK293 cells expressing HDLBP fused to a promiscuous biotin ligase BirA* (Supplementary Fig. 1d). We confirmed by RIP that BirA*–FLAG-HDLBP was also bound to several transcripts that we previously detected to be bound by the FLAG/HA-tagged HDLBP (Supplementary Fig. 4f). Reproducible identification of HDLBP-proximal proteins (Supplementary Fig. 4c, g and Supplementary Data 4) overlapped ~50 % with the previously published BioID experiment (Supplementary Fig. 4h)44 (Fig. 4d, e). We found that the top enriched proteins were involved in translation and included translation initiation factors (EIF4G1, EIF4B, EIF5, and EIF4E2) (Fig. 4d, e and Supplementary Fig. 4i), chaperones and chaperonins (HSPA1A, HSPA8, and CCT8), SRP components (SRP68) and ribosomal proteins (RPS3A and RPS10) of the small subunit (SSU). We validated the potential SSU interaction by orthogonal anti-FLAG co-immunoprecipitation experiments and found that the co-immunoprecipitates contained the SSU component of the RPS6 but very little amount of RPL7 (Fig. 4f). This finding agrees with specific 18S rRNA crosslinks detected by PAR-CLIP and HDLBP binding to an 18S rRNA sequence as shown in Fig. 3h. Taken together, these results showed that HDLBP interacts with the translational apparatus including the SRP and is localized in the proximity of ribosome-associated factors.

HDLBP promotes translation at the ER membrane. Based on previous findings45–49 and the specific interaction of HDLBP with the CDS of membrane-bound mRNAs and the ribosome observed in our study, we next addressed the function of HDLBP in ER-associated translation. We thus measured the process of active translation in the presence and absence of HDLBP by generating two CRISPR/Cas9 HDLBP knockout (KO) cell lines (Fig. 5a). The HEK293 HDLBP knockout cells showed no apparent growth defect and electron microscopy imaging of the ER revealed no morphology changes (data not shown). To quantify translation efficiency in the KO and wild-type (WT) conditions we generated ribosome profiling datasets, which showed high read periodicity and dominant in-frame P-site coverage in the CDS (Supplementary Fig. 5a, b). Since this dataset was obtained from non-fractionated cells, we asked if our experiment sufficiently captured the ribosome footprints on ER-bound mRNAs. As expected we observed low footprint density for SP and TM-containing mRNAs in the region downstream of the start codon, until the emergence of both targeting signals from the ribosome (Supplementary Fig. 5c), confirming that the data recovered ER-bound ribosome complexes with near-nucleotide resolution.

To address the differences in translation efficiency upon HDLBP KO we compared groups of membrane-bound mRNAs (Supplementary Data 5), which were classified according to the number of HDLBP crosslinks in the CDS. The highest decrease in translation efficiency was observed for mRNAs that had the largest number of HDLBP crosslinks in the CDS, suggesting that HDLBP interactions with membrane-bound mRNAs promoted translation (Fig. 5b) but did not affect ER mRNA localization (Supplementary Fig. 5d). We validated these findings by quantifying protein synthesis using pulsed stable isotope labeling with amino acids in cell culture (pSILAC)50 in combination with subcellular fractionation (Supplementary Data 6). The absence of HDLBP generally resulted in a decrease in protein synthesis of proteins encoded by membrane-bound mRNAs (Fig. 5c) and the extent of decrease depended on the level of HDLBP cross-linking signal (Fig. 5d and Supplementary Fig. 5e). Therefore, HDLBP is required for efficient protein synthesis of its target mRNAs. Since

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**Fig. 2** Differential HDLBP binding to ER-targeted and cytosolic transcripts. a Meta-transcript analysis of HDLBP cross-linking signal for cytosolic and membrane-bound mRNAs. T-C transition signal per nucleotide was normalized for library size (T-C per million). Transcripts with at least 5 T-C per million were included in this analysis. T-C per million was scaled to the maximum T-C signal per transcript. At each position, the mean scaled T-C signal was plotted. b Scatter plot of the ratio of HDLBP crosslinks in the CDS vs. 3′ UTR plotted against total crosslinks per mRNA for cytosolic and membrane-bound mRNAs. c mRNAs were split into three groups according to total HDLBP crosslinks per mRNA (n = 16: TC <0.3, membrane, n = 65: 0.3 < TC <1.39, membrane, n = 35; TC >1.39, membrane, n = 279: TC >0.3, cytosolic, n = 379: 0.3 < TC <1.39, cytosolic, n = 144: TC >1.39, cytosolic) and the distributions of the CDS vs. 3′ UTR ratio of HDLBP crosslinks were plotted. The lower and upper hinges of box plots correspond to the 25th and 75th percentiles, respectively. Upper and lower whiskers extend from the hinge to the largest or smallest value no further than the 1.5× interquartile range from the hinge, respectively. Center lines of box plots depict the median values. d Browser representation of PAR-CLIP read coverage and T-C transitions in IGF2R (membrane-bound) and HNRNPUL1 (cytosolic) mRNAs. Reads were mapped to human mRNA sequences. 5′ UTR, CDS, and 3′ UTR regions are indicated. Transcript IDs are indicated. a–d Source data are provided as a Source Data file.
the ER membrane is the primary site of translation of secretory proteins, we next asked if HDLBP influences their secretion. For this purpose, we expressed the secreted Gaussia luciferase (Gluc) and alkaline phosphatase (SEAP) in HEK293 cells in WT and KO conditions and quantified enzyme activity in the culture medium. Gluc and SEAP activity was significantly decreased upon HDLBP KO by 20–40%, showing that HDLBP depletion reduces the secretion of the two reporter proteins (Fig. 5e, f and Supplementary Fig. 5f).

Since the depletion of HDLBP reduced secretion, we next tested the impact of HDLBP overexpression. To this end we transiently transfected FLAG/HA-HDBLP into HEK293 and
HEK293 HDLBP KO cells. We also stably overexpressed HDLBP in A549 cells using a piggybac transposon carrying HDLBP (Fig. 5g).

To test the effect on secretion, we transfected the SEAP reporter construct into HEK293 and A549 cells with differing HDLBP expression levels (Fig. 5h). As expected, KO of HDLBP in HEK293 and A549 cells reduced the secretion of SEAP, whereas overexpression of HDLBP in HDLBP knockout cells rescued SEAP secretion to the levels observed in wild-type cells. Interestingly, the re-introduction of HDLBP into HEK293 and A549 cells resulted in increased secretion of about 1.15- and 2-fold, respectively (Fig. 5h) confirming that HDLBP expression levels directly influence the extent of SEAP secretion.

We next asked if the absence of HDLBP affects ribosome occupancy on membrane-bound mRNAs. For this purpose, we displayed the footprint coverage around known targeting signals (Fig. 5i and Supplementary Fig. 5g) and found that the absence of HDLBP resulted in lower ribosome density immediately downstream of the region encoding SPs or the first TM domain. This suggests that HDLBP contributes to ribosome elongation arrest, which is required for co-translational targeting and efficient translocation of the nascent peptide.

HDLBP crosslinks to CU/UU-containing codons in mRNAs and tRNAs decoding these codons. We next interrogated our ribosome profiling datasets to quantify ribosome occupancy per codon in WT and KO conditions. While we found that upon HDLBP KO several codons (including UUC/Phe, UUU/Phe, CUC/Leu, and CUU/Leu) were on average slightly more occupied by the ribosome and the P-site and E-site, this increase was not statistically significant. Therefore, global ribosome stalling in the KO condition may be measurable but could be an indirect effect of HDLBP depletion. Analysis of normalized HDLBP PAR-CLIP signal in codons (Supplementary Fig. 6a), identified the same codons (UUC/Phe, CUC/Leu, and CUU/Leu) to be among the most highly bound.

In addition, we analyzed HDLBP binding to tRNAs by PAR-CLIP enrichment as well T-C transition specificity for each tRNA cross-linking position (Supplementary Data 7) and observed that four leucine isotype tRNAs were among the top 15 enriched tRNAs in the HDLBP-bound pool (Fig. 6b). Their cross-linking sites were located in variable and D loops (Fig. 6c and Supplementary Fig. 6b) and corresponded to HDLBP mRNA binding motifs (UCUUC). Interestingly, the codons decoded by these tRNAs tend to be more occupied in HDLBP-depleted cells (Fig. 6a), suggesting that HDLBP tRNA binding enables more efficient tRNA decoding likely by facilitating tRNA recycling or reduction of tRNA diffusion from the ribosome, as proposed previously.

Absence of HDLBP results in lower proliferation and tumor formation capacity. HDLBP function in ER translation and secretion could impact the production of mitogens, growth factors, receptors and extracellular matrix, and consequently greatly influence cell proliferation, differentiation, migration, and invasion. To test this, we established a CRISPR/Cas9-induced HDLBP KO in the lung adenocarcinoma (LUAD) derived cell line A549. In 2D cultured A549 cells, adhesiveness growth was markedly (by 40–50%) reduced by HDLBP deletion (Fig. 7a). Likewise, HDLBP KO interfered with the 2D migration of A549 cells, as indicated by severely reduced wound closure in scratch migration analyses (Fig. 7b). The surface expression of the transmembrane glycoprotein CD71 encoded by the HDLBP-bound TFRC mRNA was reduced upon HDLBP KO (Fig. 7c and Supplementary Fig. 7a, b). In contrast, overexpression of HDLBP in A549 cells resulted in accelerated wound closure (Fig. 7d). Collectively, these findings suggested that HDLBP substantially influences the oncogenic properties of tumor cells. To test this in vivo, parental and HDLBP KO A549 cells were stably transduced with iRFP (infrared fluorescent protein) and viable cells were subcutaneously (s.c.) injected into athymic (FOXN1 nu/nu) nude mice to monitor tumor initiation and growth (Fig. 7e). Homogenous s.c. application of both cell populations was validated by non-invasive near-infrared imaging of iRFP immediately post-injection (Supplementary Fig. 7c, 0 days post-injection). Strikingly, tumor initiation and growth were severely reduced by HDLBP deletion (Fig. 7e and Supplementary Fig. 7c). In contrast to parental cells, which formed tumors in all eight analyzed animals, HDLBP KO cells formed palpable tumors in only three of eight animals (Supplementary Fig. 7c). In accordance, tumor volume and final tumor mass were significantly reduced by...
HDlbp deletion (Fig. 7f), indicating that HDLBP is essentially involved in tumor initiation and growth.

To determine how HDLBP KO influenced gene expression, RNA abundance was monitored by RNA sequencing in tumors (Supplementary Fig. 7d). In the absence of HDLBP, a high number of mRNAs was found to be up (n = 1039) and downregulated (n = 700), suggesting that HDLBP has a great impact on gene expression, presumably due to substantially impaired cell signaling and tumor-stroma cross-talk (Supplementary Fig. 7e and Supplementary Data 8). Strikingly, the protein products of mRNAs decreased by HDLBP KO were highly enriched for secreted proteins (e.g., collagens and matrix metalloproteases, MMP2) that function in biological processes such as “extracellular matrix organization”, “cell adhesion”, and “tissue development” (Fig. 7g). These findings supported the view, that HDLBP influences protein output, expression, and/or turnover of transcripts encoding factors essentially involved in modulating the composition of and cross-talk within the tumor-stroma landscape. To evaluate the direct control of deregulated mRNAs by HDLBP, we compared the fold changes between WT and KO xenograft tumors for subgroups of mRNAs classified according to their PAR-CLIP signal in

Fig. 4 HDLBP interacts with the translational apparatus. a PAR-CLIP coverage and crosslinks detected in pre-rRNA regions. For comparison, results for IRE1 PAR-CLIP are included. Expansion segment positions are indicated in green. b Structures of the human 80S ribosome (PDB: 4V6X) and the SRP-ribosome complex (PDB: 3JAJ) were juxtaposed and HDLBP rRNA and 7SL RNA crosslinked nucleotides were mapped (indicated in red). c RNA immunoprecipitation was performed with FLAG/HA-HDLBP as bait. Co-precipitating RNAs were detected by qRT-PCR. Average fold enrichment (anti-FLAG vs. IgG control) from four replicates was calculated with error bars indicating standard deviation. Results are shown for 7SL RNA, IGF2R, YWHAZ, ATP1A1, and CD46, as well as the mtDNA-encoded mRNA (MT-CO1) as a negative control. 

Input   FLAG    IgG

| g | 0 | 2 | 4 | 6 | 8 | 10 | 12 |
|---|---|---|---|---|---|----|----|
| log2(Dox/noDox) | 800 | 400 | 200 | 100 | 50 | 25 | 0 |
| log10(mean LFQ) | 12 | 10 | 8 | 6 | 4 | 2 | 0 |

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GO Biological process

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- Regulation of cell amide metabolic process
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HEK293 cells. We found that changes in mRNA expression of HDLBP target mRNAs (Supplementary Fig. 7f) and membrane-bound mRNAs (Fig. 7h) were significantly decreased in xenografts lacking HDLBP than for non-targets or cytosolic mRNAs. Although it remains to be addressed if HDLBP influences mRNA turnover by modulating ER-associated translation, these findings suggest that next to indirect regulation of gene expression, HDLBP also directly influences mRNA abundance, potentially by modulating ER-associated mRNA translation. In sum, the presented findings provide strong evidence that HDLBP is an important modulator of tumor progression influencing the expression of secreted proteins.
Fig. 5 HDLBP promotes ER translation and synthesis of secretory and transmembrane proteins. a Schematic overview of the ribosome profiling experiment in HEK293 parental and HDLBP KO cells. Western analysis shows the absence of HDLBP in KO cells. b Membrane-bound HDLBP target mRNAs were split into three similarly sized groups based on their cross-linking signal in the CDS (indicated in parentheses). Differences in translation efficiency (HDLBP KO vs. WT) were compared between groups. A two-sided Wilcoxon rank-sum test was used to test for significance. c 5′SLAC analysis of newly synthesized proteins in HEK293 parental and HDLBP KO cells. SILAC heavy vs. medium ratios (H/M) reflect changes in protein synthesis upon HDLBP KO and were quantified in membrane fractions. Proteins were split into three similarly sized groups based on their cross-linking signal in the CDS (indicated in parentheses). SILAC ratios were compared between groups using a two-sided Wilcoxon rank-sum test. d Proteins were split into three similarly sized groups based on their PAR-CLIP signal and membrane localization (indicated in parentheses). SILAC ratios were compared between groups using a two-sided Wilcoxon rank-sum test. e Parental and HDLBP KO cells were transfected with secreted Gaussia luciferase (Gluc) construct. Gluc activity was quantified in the medium and normalized to the intracellular Firefly luciferase (Fluc) activity. Each of the four replicate experiments (r1-r4) was carried out with five technical replicates. Data were presented as mean values ± SD. f Parental and HDLBP KO cells were transfected secreted alkaline phosphatase (SEAP) construct. SEAP signal was quantified in medium and normalized to the intracellular Firefly luciferase (Fluc) activity. Each experiment was carried out in five technical replicates. Data were presented as mean values ± SD. g Western analysis of A549 cells stably transfected with construct overexpressing HDLBP (OE). h SEAP activity, relative to Fluc, was measured in HEK293 and A549 cells with different HDLBP protein levels (KO knockout, WT wild-type, HDLBP/OE overexpression). The experiment was carried out five times with at least five technical replicates. Data were presented as mean values ± SD. i Ribosome P-site coverage around targeting signals (signal peptides and transmembrane helices) was compared between HEK293 HDLBP knockout (KO) vs. parental (WT) cells. P-site coverage was scaled to the coverage in codons 20–40 of each mRNA. A rolling mean of 5 nt was used to smooth the profiles. Absolute numbers of analyzed mRNAs are given, median signal peptide and first transmembrane helix lengths are indicated with a vertical dotted line. a–i Source data are provided as a Source Data file.

Discussion

In this work, we characterized the function of HDLBP in the context of translation at the ER. While ~80% of membrane-bound mRNAs were found to be high-affinity substrates of HDLBP (Fig. 1e), they also showed predominant binding in the CDS (Figs. 1c, 2b). In contrast, only ~40% of all cytosolic mRNAs contained HDLBP binding sites but showed significantly lower affinity and were more randomly distributed between CDS and 3′ UTRs (Fig. 2b). Investigation of the primary sequences of ER-targeted and cytosolic mRNAs revealed that the HDLBP-bound CU-containing motifs were more frequent in membrane-bound mRNAs (Fig. 3a), which also showed high codon frequency for hydrophobic amino acids, such as Leu (CUC and CUU), Ile (AUC), Phe (UUC), and Val (GUC), commonly present in signal peptides and transmembrane helices.51

While the previously reported CHHC/CHYC binding motif55 matched our findings, we further found evidence that the frequency of longer HDLBP-bound motifs up to 12 nt in length is higher than for shorter motifs in ER-bound than in cytosolic mRNAs (Fig. 3c, d). Therefore, HDLBP may have evolved to specifically recognize membrane-bound mRNAs by making use of their differential sequence composition. Multiple KH domains may allow HDLBP to recognize ER-bound mRNA through its interaction with long heterogeneous RREs and/or with multipartite motifs resulting in multivalent high-affinity binding regions, as observed for other RBPs.28,52–54 We thus quantified highly multivalent HDLBP sites and found that they correlated with high binding affinity, were ~40 nt long and contained on average 3–4 UC/CU-containing four-mers positioned several nucleotides apart. This may reflect the binding of 3–4 functional domain modules within the HDLBP protein structure to multiple RREs. Using in vitro binding assays (Fig. 3i), we confirmed these observations and conclude that multivalent interactions indeed result in the higher affinity of HDLBP to long RNA-binding regions. Further studies will provide insight into which domains or their combinations are responsible for the recognition of long multivalent sites and the formation of HDLBP-containing mRNPs. The recently identified C/U-repeat-containing SECReTE motif7 supports the existence of long functional sequences in yeast mRNA that are required for the secretion of encoded proteins and very likely represents the RNA-binding sites of the HDLBP yeast orthologue Sdp160p.56

In addition, we report that HDLBP is interacting with ER-associated mRNAs and promoting their translation resulting in increased secretion of the synthesized proteins (Fig. 5), which is in line with previous findings.34,35 Since we found high-affinity HDLBP binding sites not only in ER-targeted mRNAs but also in membrane-localized mRNAs with no known or predicted targeting signals (Fig. 1d), HDLBP may significantly contribute to the efficient translation of both SRP-dependent and -independent mRNAs.8–11 Profiling of SRP-dependent and -independent mRNAs in mammalian cells will contribute to this understanding in future studies.

Furthermore, we show that HDLBP absence results in decreased ribosome elongation arrest around targeting signals (Fig. 5g). This process is required for efficient targeting and translocation of nascent peptides to the ER lumen, as well as to prevent misfolding, aggregation, and ER-associated degradation.55 HDLBP-mediated local ribosome slowdown may thus promote targeting, translocation, and accurate folding of protein domains encoded by HDLBP-bound regions. This possibility is strongly supported by the detected h16 and ES65B 40S HDLBP interactions, which are in proximity to the EEF2 and EIF4G2 binding sites41 and could influence ribosome elongation arrest. In addition, low-affinity HDLBP contacts detected in the 7SL RNA region, which is required for the delay in GTP hydrolysis during elongation arrest, and membrane targeting activity also support this notion. Finally, we identified key chaperones and chaperonins in close proximity to HDLBP, supporting its role in protein folding, as previously suggested for its yeast orthologue.36

Mechanistically, we propose that HDLBP interacts with the translational apparatus by binding to the mRNA and the small ribosomal subunit as well as to other ribosome-associated factors. High-affinity mRNA binding contributes to the formation of elongation-arrested cytosolic RNCs, allowing their efficient localization to the ER membrane, translocon handover, and/or enabling proper folding of the nascent peptide. Since HDLBP is most likely bound to the mRNA downstream from the elongating ribosome, it could be sequestered from the mRNA via ribosome collisions possibly via TRNA and/or ribosome ES65B-dependent mechanism, which remains to be elucidated. We speculate that HDLBP is bound to the mRNA only during the primary round of translation after which it is removed. This step may allow the mRNA molecule to localize to the ER, where additional rounds of
translation can be carried out in the presence of ER folding machinery and aggregation quality control. In the future, the sequence of HDLBP binding events to mRNA, rRNA, and tRNA during different stages of translation should be addressed.

In the absence of HDLBP, we also detected increased ribosome occupancy at P- and E-sites. Since this increase was modest and not statistically significant, it may be a consequence of the indirect effects of HDLBP KO. Nevertheless, several studies suggest that E-site may act as a sensor for ribosome elongation kinetics and that E-site occupancy may directly influence translation fidelity and ribosome translocation, which could be another possible mechanism of how HDLBP promotes translation elongation.
Methods

Cell lines and culture conditions. HEK293 Flp-In T-REX (HEK293) (Thermo Fisher Scientific), HEK293 stable cell lines, and A549 cells were cultured in standard Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% l-glutamine (200 mM, Thermo Fisher Scientific). Stable HEK293 cell lines expressing HDLBP FLAG/HA or BirA*-FLAG-HDLBP were generated by hygromycin selection. Induction of the stable cell lines was achieved by adding 1 μg/ml of doxycycline to the culture medium and incubation for 16 h.

HEK293 and IG9 HDLBP knockout cell lines were produced using the Edit-R CRISPRi Knockout Kit (Dharmacon) according to the manufacturer’s instructions. Briefly, transfections of synthetic tracrRNA (U-002000-05), hCMV-Puro-R-Cas9 (U-005100-120), and pre-designed HDLBP crRNA (either guide 1 (CR-019956-01-0005) or guide 2 (CR-019956-04-0005)) or a non-targeting control (U-007501-05) were carried out using DharmaFECT Duo transfection reagent (Dharmacon, T501001) in a 12-well plate. After 2 days cells were seeded to a 10 cm dish and treated with puromycin (2 μg/ml for HEK293 cells and 1 μg/ml for A549 cells). The surviving colonies were picked and Western analysis was performed.

A stable A549 cell line expressing HDLBP was generated by co-transfection of PB-TAG-ER221-PB and pCMV-hyPhase2 in a 12-well plate using Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer’s instructions. A puromycin selection was carried out and induction of the stable cell line was achieved by adding 1 μg/ml of doxycycline to the culture medium.

Plasmids. Vector pDONR221 carrying the HDLBP coding sequence was obtained from the hORFeome V5.1 collection and recombined into pFR7/T-OFLAG/HA-Dest (Addgene ID: 26360), pDEST-BirA-FLAG-N-term-pcDNA5-FRT-Pu and pShuttle-TAG-ER221 (Addgene ID: 80479) using the Gateway LR Clonase II (Thermo Fisher Scientific) according to the manufacturer’s protocol. To purify recombinant full-length HDLBP, we amplified HDLBP from pENTR221 HDLBP with the primers HDLBP_C_fwd, BglII and HDLBP_end_rev_NotI (see Oligonucleotides) and ligation into BglII and NotI restriction sites in pLingK. To purify the HDLBP protein variant D, we amplified variant D from pENTR221 HDLBP with the primers HDLBP_D_fwd, BamHI and HDLBP_kh9_rev_NotI (see Oligonucleotides) and ligation into BamHI and NotI restriction sites in pLingK. For the SEAP secretion assays, we transfected pEXZ-GA01 (GeneCopoeia) and additionally pFRTpsiCHECK containing Renilla luciferase and firefly luciferase. For the SEAP rescue secretion assays we replaced Renilla luciferase in pFRTpsiCHECK with SEAP luciferase by amplification from pEXZ-GA01 with the primers SEAP_fwd and SEAP_rev (see Oligonucleotides) and ligation into Nhel and NotI restriction sites. To carry out the Gusia luciferase secretion assay, we replaced Renilla luciferase in pFRTpsiCHECK with Gaussia luciferase by amplification from pEXZ-GA01 with the primers Gaussia_fwd and Gaussia_rev (see Oligonucleotides) and ligation into Nhel and NotI restriction sites. Vectors have been submitted to Addgene.

BioloX proximity ligation assay. The BiolOx proximity ligation assay was performed as described before 66 with minor modifications. Stable cell lines expressing BirA*-FLAG-HDLBP (four 15 cm dishes per replicate) were incubated in the absence or presence of 1 μg/ml doxycycline for 24 h. Next, 250 μg biotin was added for 3.5 h. Cells were washed four times with PBS, harvested, snap-frozen in liquid nitrogen, and stored at −80 °C. Cell pellets were incubated with 3 ml RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% IGEPAL CA-630, 1% EDTA, 0.1% SDS, 0.5% sodium deoxycholate, complete EDTA-free protease inhibitor cocktail [Roche]) for 1 h on ice at slow agitation. Cell lysates were cleared (15,000 × g, 15 min, 4 °C) and filtered through a 5 μm Supor membrane. To wash streptavidin sephrose (GE Healthcare, 17-5113-01) the suspension was centrifuged at 400 × g for 1 min, the supernatant was removed and 1 ml RIPA buffer with (0.5%) sodium deoxycholate and without complete EDTA-free protease inhibitor cocktail was added. The wash step was repeated two times. The RIPA buffer was removed and 40 μl per replicate of sephrose was added to the sample. After 3 h incubation at 4 °C on a rotating wheel the sephrose was washed twice with RIPA buffer, twice with TAP lysis buffer (50 mM HEPES-KOH [pH 8.0], 100 mM KCl, 2 mM EDTA, 1% IGEPAL CA-630) and three times with 50 mM ammonium bicarbonate. About 90% of the sample was stored at −80 °C and further processed for mass spectrometry.

Mass spectrometry BioLoX. Beads were resuspended in 200 μl of 50 mM ammonium bicarbonate containing 2 μl trypsin (Promega, V511A). The samples were incubated for 16 h in a Thermomixer (Eppendorf) at 37 °C and 750 rpm shaking. Afterwards, 1 μg of trypsin was again added and the samples were incubated further for 2 h. Samples were then centrifuged at 400 × g for 2 min and the supernatant was transferred to a new vial. To ensure complete beads removal, the samples were centrifuged at 16,000 × g for 10 min and the supernatants were transferred to a new vial containing 5 μl of trifluoroacetic acid. Each sample was loaded on two StageTips for desalting, Eluates for each sample were pooled together prior to MS analysis.

For all the samples, 5 μl were injected in duplicate on a LC-MS/MS system (EksperNanoLC 415 [Eksigent] coupled to Q-Exactive HF [Thermo]), using a 240 min gradient ranging from 2 to 45% of solvent B (80% acetonitrile, 0.1% formic acid; solvent A = 5% acetonitrile, 0.1% formic acid). For the chromatographic separation, the nanospray source was operated with a spray voltage of 2.3 kV and an ion transfer tube temperature of 250 °C. Data were acquired in data-dependent mode, with a top ten method (one survey MS scan with resolution 60,000 at m/z 200, followed by up to ten MS/MS scans on the most intense ions, resolution 15000, intensity threshold 5000). Once selected for fragmentation, ions were excluded from further selection for 30 s, in order to increase new sequencing events.

Fig. 6 HDLBP crosslinks to tRNAs decoding CU/UU-containing codons. a Difference in codon frequencies in the P-site (top) and E-site codons (bottom) in HDLBP KO vs. WT. Mean codon shift was calculated for four replicates (mean ± standard deviation is shown). b Enrichment of tRNAs in HDLBP PAR-CLIP and their binding sites. T-C transitions in tRNAs were normalized to total RNA abundance and ranked from highest to the lowest value (left to right). For each T-C transition, we displayed its transition specificity (T-T transition vs. total read coverage). Mean values of two PAR-CLIP biological replicates ± SD are depicted. Total log2-transformed RNA abundance and codon usage are also shown (top). c (Left) Browser representation of alignment to RNA Lew-UAG. T-C transitions in the D-loop and V-region are indicated for the HDLBP PAR-CLIP dataset. The second track shows coverage in the total RNA sample. (Right) HDLBP crosslinked uridines are indicated with respect to secondary RNA structure.

Overall, our results point to a general function for HDLBP in ER translation. Although this is obviously important for every cell, we expect that this phenotype is most prominent in specialized secretory cell types (e.g., fibroblasts, pancreatic and immune cells). In support of this view, HDLBP mRNA expression, and thus likely HDLBP protein level is substantially elevated in secretory cells (Supplementary Fig. 7g), most prominently in fibroblasts, which are key to orchestrating the extracellular landscape by producing and remodeling the extracellular matrix. In addition, HDLBP misregulation gives rise to far-reaching consequences and modulation of disease phenotypes, such as impaired viral replication, atherosclerotic plaque formation, and autism. Finally, the results presented in this study highlight the striking involvement of HDLBP in lung tumor cells during tumor progression and suggest that therapeutic interventions targeting HDLBP may represent a previously unrecognized strategy for inhibiting lung tumor growth or other malignancies. In the future, further implications of the regulatory role of HDLBP for tumor biology need to be explored.
modification, oxidation of methionine, and acetylation of N-terminus were chosen as variable modifications. The search engine peptide assignments were filtered at 1% FDR and the feature match between runs was not enabled; other parameters were left as default.

Mass spectrometry proteomics data have been deposited to ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD018313.

**Western analysis.** Cell pellets were lysed directly in Laemmli buffer, sonicated (5-s-pulse at 80% amplitude), and centrifuged (10,000 × g, 10 min, 4 °C). Before resolving the proteins by SDS-PAGE samples were boiled for 3 min (95 °C). For transferring the proteins to a nitrocellulose membrane (Whatman) a semi-dry blotting apparatus (20 V for 1 h) was used. The membranes were blocked with 5% non-fat milk for 1 h and incubated with the primary antibody overnight (anti-HDLBP (Abcam, ab109324; dilution 1:10000), anti-beta-Tubulin (Sigma, T8328);...
diluted 1:5000, anti-HA (Covance, MMS-101P-100; diluted 1:5000), anti-BCAP31 (ProteinTech, 11200-1-AP; diluted 1:2000), anti-RPS6 (Cell Signaling, #2217; diluted 1:1000), and anti-RPL7 (Abcam, ab72550; diluted 1:5000). After washing the membranes three times in TBST (150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.1% Tween 20) the membranes were incubated with 1:4000 dilution of horseshad pad peroxidase-conjugated secondary antibodies (goat anti-rabbit immunoglobulins/HRP—Agilent cat# P044801-2 and goat anti-mouse immunoglobulins/HRP—Agilent cat# P044701-2) for 2 h. The membranes were washed three times with TBST, bands were visualized with ECL detection reagent (GE Healthcare), and imaged with an ImageQuant LAS-4000 imaging system or an Amersham Image 680 imaging system (GE Healthcare).

Cell fractionation. Cell fractionation by sequential detergent extraction was performed as previously described with minor modifications. HEK293 and HEK293 HDLBP knockout cell lines (one 15 cm dish per replicate) were grown to ~90% confluency. Cells were washed with PBS. All further steps were carried out on ice using ice-cold reagents and cells were always pelleted at 3000 × g for 5 min at 4°C. First, PBS containing 50 μg/ml cycloheximide was added for 15 min at 4°C on a rotating wheel. Afterwards the supernatant was collected as the cytosolic fraction. To wash the pellet, 5 ml of washing buffer (110 mM KOAc, 25 mM K-HEPES [pH 7.2], 2.5 mM Mg(OAc)₂, 1 mM EGTA, 0.015% digitonin, 1 mM DTT, 50 μg/ml cycloheximide, complete EDTA-free protease inhibitor cocktail [Roche], 40 μU/ml SUPERaseIn [Thermo Fisher Scientific]) per sample and incubated with three cell pellet volumes of lysis buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.05% IGEPAL CA-630, 0.5 mM DTT, complete EDTA-free protease inhibitor cocktail [Roche]) three times and a second RNase T1 treatment was carried out. For PAR-CLIP experiments, the second RNase T1 digestion was carried out with 50 μg/ml cycloheximide and 0.5 mM DTT.

Next generation sequencing was carried out on a HiSeq 2500 Illumina instrument (1 × 51 ± 7 cycles).

Ribosomes profiling library preparation. Ribosome profiling was performed as described previously with minor modifications. HEK293 and HEK293 HDLBP knockout cell lines (one 10 cm dish per replicate) were grown to ~90% confluency. Cells were washed with ice-cold PBS containing 100 μg/ml cycloheximide. The PBS was then replaced with FBS-free media, and plates were put on liquid nitrogen for 10 s and subsequently on ice. About 400 μl mammalian polysome buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.5 μg/ml cycloheximide, 1% Triton X-100, and 25 U/ml TurboDnase [Thermo Fisher Scientific]) per plate was added. Cells were scratched and collected, and the lysates were passed ten times through a 26 G needle. After clearing the cell lysates by centrifugation (20,000 × g, 10 min, 4°C) 120 μl cell lysate aliquots were snap-frozen and stored at ~80°C. One aliquot of cell lysate was used for RNA sequencing (see RNA sequencing library preparation) and therefore RNA was isolated using Trizol LS (Thermo Fisher Scientific) in combination with RNA Clean & Concentrator-25 kit (Zymo Research). Another aliquot of cell lysate was used to isolate the ribosome-protected fragments by adding 300 U RNase I (Thermo Fisher Scientific) for 45 min at room temperature at slow agitation. Meanwhile, MicroSpin S-400 HR Columns (GE Healthcare) were equilibrated by adding regularly cold mammalian polysome buffer (without DTT, cycloheximide, Triton X-100, TurboDnase) to the columns. The columns were centrifuged (6000 × g, 4 min, 4°C). About 100 U SUPERaseIn (Thermo Fisher Scientific) per sample was added, mixed, and subsequently, the cell lysates were applied dropwise to the columns (100 μl cell lysate per column). The columns were centrifuged (6000 × g, 2 min, 4°C) and the flow-through was collected. RNA was isolated using Trizol LS (Thermo Fisher Scientific) in combination with RNA Clean & Concentrator-25 kit (Zymo Research). The ribosome-protected fragments were then depleted with the RiboZero Kit (Illumina) according to the manufacturer’s protocol by using 5 μg RNA as input. The remaining RNA was separated on a 17% denaturing urea-PAGE gel (Carl Roth) and RNA fragments in the range from 27 to 30 nt were excised (detected by markers (see Oligonucleotides)). Sequencing libraries were generated as described in the PAR-CLIP section. Next-generation sequencing was carried out on a HiSeq 2500 Illumina instrument (1 × 51 ± 7 cycles).

Pulsed SILAC. HEK293 and HEK293 HDLBP knockout cell lines were cultured for at least three passages in arginine- and lysine-free DMEM (Life Technologies)

References
containing 10% dialyzed PBS (PAN-Biotech), 1% glutamax (Life Technologies), 1% sodium pyruvate (Life Technologies), and “light” form amino acids 0.2 mM l-arginine (Sigma-Aldrich) and 0.8 mM l-lysine (Sigma-Aldrich). Cells were seeded in six-well plates (450,000 cells per well). After 48 h the “light” form medium was removed. It was replaced by either medium containing the “medium” form amino acids (l-[13C6]-arginine (Sigma-Aldrich), l-[2-15N4]-lysine (Cambridge Isotope Laboratories), l-[13C6,15N4]-lysine (Cambridge Isotope Laboratories)) and cell fractionated after 2 h or 4 h. Cell fractionation was carried out as described above with reduced volumes: six wells were washed with cold PBS. About 544 μl PBS containing 50 μg/ml cycloheximide was added for 10 min. In the meantime, cells were split and washed. The heavy and medium SILAC labelled cells were combined in an Eppendorf tube. Downstream 100 μl of permeabilization buffer, 544 μl of wash buffer, and 100 μl of lysis buffer were used. After clarification of the fractions 90 μl sample was recovered and 810 μl pure EtOH was added and samples were submitted to mass spectrometry.

Mass spectrometry pulsed SILAC. Protein samples were resuspended in 6 M urea, 2 M Thiourea, and 10 mM HEPES pH 8 solution. Proteins were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide at room temperature. For lysis, proteins were incubated with 1% (v/v) llysyl endopeptidase (Wako) at room temperature for 3 h; diluted with 50 mM ammonium bicarbonate solution for a final urea concentration of 2 M, and incubated with 1% (v/v) trypsin (Promega) under constant agitation at room temperature for 16 h. Peptides were acidic with 1% (v/v) trifluoroacetic acid and desalted with C18 StageTips. Prior to LC-MS/ MS analysis, peptides were digested with 50% acetonitrile 0.1% formic acid, dried, and resuspended in 3% acetonitrile, 0.1% formic acid (Buffer A). The peptide concentration was measured based on 280 nm UV light absorbance.

Reversed-phase liquid chromatography was performed employing an EASY nLC II (Thermo Fisher Scientific) using self-made C18 microcolumns (75 μm ID, packed with ReproSil-Pur C18-AQ 1.9-μm resin, Dr. Maisch, Germany) connected online to the electrospray ion source (Proxeon, Denmark) of both an Orbitrap HR-X or an Orbitrap Elite mass spectrometer with the FAIMS module installed and in application mode “Peptide” (Thermo Fisher Scientific). Peptides were eluted at a flow rate of 250 nL/min over 2 or 4 h with a stepwise increasing gradient of 4.74 to 81.3% acetonitrile in constant 0.1% formic acid. Settings for data-dependent analysis on Orbitrap HR-X were: positive polarity, full scan (resolution, 60,000; m/z, 350–1800), injection time of 30 ms, and 200/200–2000/200 MS/MS analysis; peptides were sequenced with 50% acetonitrile 0.1% formic acid, dried, and resuspended in 3% acetonitrile, 0.1% formic acid (Buffer A). The peptide concentration was measured based on 280 nm UV light absorbance.

between different classes of proteins were compared by cumulative density function and significance was evaluated by the Wilcoxon rank-sum test.

Cell proliferation and migration assays. The formation of cell proliferation, 1 x 10⁴ Ctrl or HDLBP KO A549 cells were seeded in 96-well plates. Cell counting was monitored for 48 h with a Live/Dead assay with 4× magnification for a whole well scan. Confluent masks were generated using the IncuCyte analysis software. Cell viability and DNA content were determined using
CellTiter Glo (Promega) supplemented with 1/2000 SYBR Green 1 (Thermo Fisher) according to the manufacturer’s protocols. Luminescence and fluorescence intensities were measured using a Tecan Infinite M200pro microplate reader (Tecan). For the scratch wound migration analysis, 2.5 x 10⁴ Ctrl, HDLBP KO A549 cells and stable A549 cells expressing HDLBP (±doxycline) were seeded in a 96-well Imagent plate (Sartorius) for 24 h. The wound areas were created on confluent cell monolayers using a 96-well WoundMaker (Sartorius). Wound healing was monitored using an IncuCyte S3 system at 10x magnification and 1 h intervals. Confocal and wound masks were generated and quantified using the IncuCyte analysis software.

Flow cytometry. To determine changes in CD71 expression and presentation, 2 x 10⁶ HDLBP knockout and WT control cells were seeded in six-well plates and grown for 2 days. After harvesting using trypsin, cells were counted. For CD71 surface labeling, 3 x 10⁵ cells were stained with anti-CD71 antibodies (human CD71, clone 77A10; APC-conjugated; Miltenyi Biotec; RID-AB 2660542; dilution 1:111) or isotype control (REA control antibodies, clone AE293, APC-conjugated; Miltenyi Biotec; RRD-AB 7733447; dilution 1:50) diluted in 1% BSA/PBS for 15 min at 4°C. After washing with PBS, cells were analyzed by flow cytometry using a MACSQuant Analyzer (Core Facility Imaging; Martin-Luther University Halle-Wittenberg). Dead cells were excluded by propidium iodide (Miltenyi Biotec) staining. Mean fluorescence intensities of CD71 were determined upon background subtraction using the FlowJo analyses software. The experiment was carried out three times.

PAR-CLIP data processing and analysis. Reads were demultiplexed, stripped of the 3’-adapter sequence by Flexbar (v2.5), and collapsed to remove PCR duplicates. This was followed by trimming of four nucleotides from both the 5’ and 3’ end of the reads and the reads were mapped using a human genome (hg19 build) using BWA v0.7.15-r1140 and the previously published computational PAR-CLIP pipeline (v0.97a) [25] https://github.com/marvin-jens/clip-analysis. Briefly, read clusters were called from unique alignments and scored for characteristic T-C transitions. After false positive filtering (using antisense clusters that decreased in size and a false discovery rate of 0.05) the remaining clusters were written as bed files. Clusters obtained from each biological replicate were additionally filtered for reproducibility. We only considered those clusters that overlapped by at least 50% of their nucleotide length between replicates. In addition, we required that the positions of the highest T-C transition values per cluster were no more than ten nucleotides apart between two replicates. Each cluster was also required to have at least three or more mean T-C transitions calculated between replicates. To obtain gene-level binding information, we summed T-C transitions in reproducible clusters for each gene. We thus obtained the total number of crosslinks positions within whole mRNAs, or within the CDS, 5’ UTR, or 3’ UTR. To calculate for PAR-CLIP expression level bias, we divided the total number of crosslinks per gene by the corresponding TPM value as obtained by RSEM (v1.1.2) from our RNA-seq experiment from unfractonated HEK293 cells (see RNA-seq description below). This provided us with PAR-CLIP enrichment values (Fig. 1d, f).

To assess the relative distribution of T-C transitions within the transcriptome and obtain accurate mapping to transcripts originating from repetitive genomic loci, reads were sequentially mapped to reference transcripts by Bowtie2 (v2.2.2) in the following order by retaining the unmapped reads from the previous to the next mapping step. We started with human pre-rRNA (GenBank U13369.1), followed by rRNA (GenBank NR_030387.2, NR_030388.1, NR_030389.1, NR_030390.1, NR_030383.1, NR_030384.1), snRNA, snoRNA, other ncRNA (all from Ensembl, including RN17S1), tRNA (GtRNADb), mtDNA (GenBank AF347015.1), and finally the human genome (hg19, gEnomes). The last genome-mapping step was performed by the STAR aligner (v2.2.1) where only uniquely mapped reads (MAPQ ≥ 20 or more and T-C transitions were extracted using bowtie2 MAPQ = 255) were retained. Except for the RNA mapping (see below for details), we retained reads that mapped with a Bowtie2 MAPQ = 20 or more and T-C transitions were extracted using row_outpipe_coverage_plus_T-C.pl script [26]. For transcriptome level analysis, remaining reads after mdmA mapping were aligned to the transcriptome sequence (GTFF annotation file Gencode v19) by the STAR (v2.5.3a) by retaining only the reads that uniquely mapped to the hg19 genome (parameters -outFilterMultimapNmax 1 -outFilterMismatchNmax 1 -outFilterMismatchNmin 15 -alignMismatchesMinN 5 -seedSearchStartLmax 20 -outFilterOverhangMin 30 8 8 -quantMode TranscriptomeSAM). Transcriptome BAM files were converted to the bed format using BedTools bamToBed (v2.26.0). Bed files were then input into riboWaltz (v1.1.0) [27], which we used for downstream quality control and P-site coverage analysis.

Ribosomal profiling data processing and analysis. Reads were demultiplexed and adapter sequences were removed by Flexbar (2.5). Reads were then collapsed to remove PCR duplicates, followed by the removal of random nucleotides (four on both the 5’ and 3’ end of the reads) using fasts_trimmer (FASTX Toolkit 0.11.4). Reads aligning to RNA sequences and other sources of contamination using a custom human genome were removed by Bowtie2 (v1.1.2) as transcriptome alignment program. Read counts were normalized using the corresponding TPM value as obtained by the DESeq2 RNA-seq library preparation, data processing, and analysis. RNA sequencing libraries were prepared using the TruSeq Stranded mRNA kit (Illumina) according to the manufacturer’s protocol. For the Illumina library, we used 1 μg RNA as input. RNA-seq sequencing was carried out on a HiSeq 4000 or NextSeq 500 Illumina instruments. Reads were demultiplexed and 3’-adapter sequences removed by Flexbar (v2.5). Read counts per gene and TPM values were obtained by RSEM (v1.1.2) [28] using default parameters and Bowtie (v1.1.2) [29] as transcriptome alignment program. Read counts and TPM values were normalized using the corresponding TPM values between and within fractions were performed using standard parameters. Log2-transformed fold changes were obtained from the DESeq2 output. Membrane-to-cytosol enrichment was calculated as the log2-transformed fold change between the membrane and cytosolic samples. To define membrane-bound and cytosolic nucleotide coverage of five was requested in at least one of the samples. To perform the metagenase analysis of P-site occupancy across the start and stop codons (Supplementary Fig. 5c), we performed the membrane subtraction of PAR-CLIP expression level bias, which provided normalized P-site coverage per nucleotide. To include a P-site in the downstream metagenase analysis, a minimal P-site nucleotide coverage of five was used in at least one of the samples.

To perform the metagenase analysis of P-site occupancy across the start and stop codons (Supplementary Fig. 5c), we excluded the P-site coverage corresponding to the first two and the last two codons of the coding sequence. The reason for this is the high read coverage at these positions due to ribosome initiation and termination which would provide artificial normalization, which would confound the differences in the regions around the start and stop codons. P-site coverage per nucleotide was first normalized for library size and summed for each codon. Next, the codon coverage was scaled to the mean CDS coverage excluding the coverage at the extremities. We included all codons that had total codon coverage of five or higher and all interrogated codon positions within the CDS, the scaled P-site coverage was averaged. A rolling mean over 10 nt was used to smooth the signal in the final metagenome plot. To perform the metagenase analysis of codon occupancy surrounding targeting signals (Fig. 5c and Supplementary Fig. 5g), we summed the P-site coverage per codon. The obtained codon coverage was normalized to the mean coverage within codons 20–40 in each codon sequence, as described previously [30]. For all interrogated codon positions within the CDS (1–500), the scaled P-site coverage was then averaged over all well-quantified transcripts (mRNAs with TPM ≥ 10). A rolling mean over 5 nt was used to smooth the signal in the final metagenome plot. To calculate translation efficiencies per the gene, we used RSEM (v1.2.2) which supplied us with read counts and TPM values per gene. Differences in translational efficiency, as well as in mRNA abundance and due to both effects (transcription and translation) were detected by DESeq2 (1.18.1) with an interaction term model as described previously [31]. Briefly, RFP read counts were normalized using the DESeq2 estimateSizeFactors function by considering all read counts. DESeq2 was run with default parameters. Log2-transformed fold changes for downstream comparisons were taken directly from the DESeq2 output. For codon level analysis, we used the codon frequency analysis used by riboWaltz. Differences in codon frequency were obtained by subtracting the codon occupancy values between conditions and calculating standard deviations between replicates.

Targeting signal annotations. Signal peptide and transmembrane helix annotations were downloaded from Ensembl Biomart (http://grch37.ensembl.org/biomart/martview), which uses SignalP [32] and TMHMM [33] for annotation.
ncRNA-seq library preparation, data processing, and analysis. For normalization of the ncRNA abundance in the HDLBP PAR-CLIP dataset we made use of the RNA-seq library preparation protocol based on small RNA cloning approaches. Total RNA was extracted from HEK293 cells with Qiagen RNAeasy kit according to the manufacturer’s instructions. For each sample, we extracted 10 μg of RNA and subjected it to oligo(dT) purification using Qiagen Oligo(dT) beads according to the manufacturer’s instructions. The RNA was then heated at 65 °C for 5 min, snap-frozen in liquid nitrogen, and stored at −80 °C. Next, we determined the concentrations of the RNA samples using a Nanodrop spectrophotometer (Thermo Scientific NanoDrop 1000) and quality checked them using an Agilent 2100 Bioanalyzer (A260/A280 ratio > 1.8). The RNA was then purified using a Qiagen RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. After purification, the RNA was treated with DNase I (Thermo Scientific TURBO DNase) to remove any genomic DNA contamination. The RNA samples were then reverse transcribed using the SuperScript IV Reverse Transcriptase (Thermo Scientific) and random hexamers according to the manufacturer’s instructions. The cDNA was then amplified by PCR using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and primer pairs specific for the 5′ end of the cDNA library and the 3′ end of the cDNA library. The amplification was performed in a total volume of 100 μl containing 1x Phusion buffer, 2 mM dNTPs, and 1 μM of each primer. The PCR was carried out for 30 cycles with an initial denaturation step at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s, and a final extension step at 72 °C for 5 min. The PCR products were then purified using a Qiagen PCR Purification Kit (Qiagen) according to the manufacturer’s instructions. The purified cDNA was then fragmented using sonication to obtain fragments of approximately 200 bp with an average size of 220 bp. The fragmented cDNA was then cleaned up using a Qiagen RNeasy Mini Kit (Qiagen) and eluted in 100 μl of water. The fragmented cDNA was then fragmented using sonication to obtain fragments of approximately 200 bp with an average size of 220 bp. The fragmented cDNA was then cleaned up using a Qiagen RNeasy Mini Kit (Qiagen) and eluted in 100 μl of water.

For each sample, we performed the three-step library preparation process as described above. The RNA samples were purified using a Qiagen RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. After purification, the RNA was treated with DNase I (Thermo Scientific TURBO DNase) to remove any genomic DNA contamination. The RNA samples were then reverse transcribed using the SuperScript IV Reverse Transcriptase (Thermo Scientific) and random hexamers according to the manufacturer’s instructions. The cDNA was then amplified by PCR using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and primer pairs specific for the 5′ end of the cDNA library and the 3′ end of the cDNA library. The amplification was performed in a total volume of 100 μl containing 1x Phusion buffer, 2 mM dNTPs, and 1 μM of each primer. The PCR was carried out for 30 cycles with an initial denaturation step at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s, and a final extension step at 72 °C for 5 min. The PCR products were then purified using a Qiagen PCR Purification Kit (Qiagen) according to the manufacturer’s instructions. The purified cDNA was then fragmented using sonication to obtain fragments of approximately 200 bp with an average size of 220 bp. The fragmented cDNA was then cleaned up using a Qiagen RNeasy Mini Kit (Qiagen) and eluted in 100 μl of water. The fragmented cDNA was then fragmented using sonication to obtain fragments of approximately 200 bp with an average size of 220 bp. The fragmented cDNA was then cleaned up using a Qiagen RNeasy Mini Kit (Qiagen) and eluted in 100 μl of water.

For each sample, we performed the three-step library preparation process as described above. The RNA samples were purified using a Qiagen RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. After purification, the RNA was treated with DNase I (Thermo Scientific TURBO DNase) to remove any genomic DNA contamination. The RNA samples were then reverse transcribed using the SuperScript IV Reverse Transcriptase (Thermo Scientific) and random hexamers according to the manufacturer’s instructions. The cDNA was then amplified by PCR using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and primer pairs specific for the 5′ end of the cDNA library and the 3′ end of the cDNA library. The amplification was performed in a total volume of 100 μl containing 1x Phusion buffer, 2 mM dNTPs, and 1 μM of each primer. The PCR was carried out for 30 cycles with an initial denaturation step at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s, and a final extension step at 72 °C for 5 min. The PCR products were then purified using a Qiagen PCR Purification Kit (Qiagen) according to the manufacturer’s instructions. The purified cDNA was then fragmented using sonication to obtain fragments of approximately 200 bp with an average size of 220 bp. The fragmented cDNA was then cleaned up using a Qiagen RNeasy Mini Kit (Qiagen) and eluted in 100 μl of water. The fragmented cDNA was then fragmented using sonication to obtain fragments of approximately 200 bp with an average size of 220 bp. The fragmented cDNA was then cleaned up using a Qiagen RNeasy Mini Kit (Qiagen) and eluted in 100 μl of water.
MgCl₂, 0.5 mM TCEP, and eluted with 50 mL of buffer containing 50 mM Tris-HCl pH 8.0 and 0.2 M NaCl. The eluted protein was supplemented with 5 mM thiophosphate overnight at 37 °C to phosphorylate at 37 °C in 1× T4 polynucleotide kinase buffer supplemented with 0.5 mM ATP-γ-S, 5 mM DTT, and 1 unit of T4 polynucleotide kinase followed by ethanol precipitation. Fluorescin was then added to the 5’ end of the RNA by incubating the RNA with 1 mM fluorescein maleimide for 2 h at room temperature in the dark in 50 mM phosphate buffer pH 7.0 followed by ethanol precipitation. The fluorescence polarization assay was performed as described before with minor modifications. Briefly, serial dilutions of either full-length HDLBP or its various fragments with N-terminal GST-tag in 1× binding buffer (20 mM Tris, pH 7.5, 60 mM KCl, 1 mM EDTA, 10% glycerol, 1 ng/μg tRNA, 1 ng/μl heparin, 0.4 U/μl RNasin, and 200 ng/μl BSA) were first added to the wells of a black 384-well flat-bottomed microplate (Corning® NBS) followed by addition of the fluorescein-labeled RNA probes to a final concentration of 10 nM. The final reactions were mixed by a microplate shaker, spun, and were incubated on ice for 20 min in the dark. The anisotropy values were measured and automatically calculated by the fluorescence polarization function of microplate reader SpectraMax iD5 (Molecular Devices), using the SoftMax® Pro 7.2 software.

Live single-molecule imaging and ER co-localization analysis. Imaging and image data analysis were performed analogously to the protocols described before. In brief, HDLBP KO/Crls were generated in HeLa 11ht cell lines expressing Gaussia luciferase reporter transcripts from a single genomic location. Reporter transcript expression was induced by the addition of doxycycline for 1 h. Images were acquired 1–2 h after removal of doxycycline from the medium. The MS2-stem-loop encoding reporter transcripts were detected via MS2 coat protein-Halo fusion proteins that were labeled with Janelia dye JF549 and stably co-expressed from the HeLa cell genome. The ER was detected using a Turq2-KDEL ER marker that was also stably expressed in the cells. The image series consisted of 100 frames that were acquired at frame rates of 20 Hz (50 ms exposure). Single-particle mobility and ER co-localization analysis included all tracks obtained from single-particle tracking that were longer than 2 frames.

Oligonucleotides. Oligonucleotides are listed in Supplementary Table 1 in Supplementary Information.

Data availability
The data supporting the findings of this study are available from the corresponding authors upon reasonable request. PAR-CLIP, RNA-seq, and ribosome profiling data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO) under accession GSE148262. BioID and pSLAC data have been submitted to the ProteomeXchange under the dataset identifiers PXD018313 and PXD018316, respectively. Source data for the figures and supplementary figures are provided as a Source Data file.

Code availability
All analysis scripts and processed data are publicly available from GitHub at https://github.com/mmilek/hdlbp_rev.git (https://doi.org/10.5281/zenodo.6347386).

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Author contributions
U.Z., M.M. and M.L. conceived the study and designed experiments. U.Z. performed the majority of the experimental work, analyzed data, and prepared figures. M.M. carried out the majority of the computational data analysis and prepared the majority of figures including Supplementary Material. I.M. conducted FP assays and was supervised by M.L. C.H.V.-V. contributed psILAC mass spectrometry experiments and was supervised by M.S. G.M. and S.K. contributed to BioID mass spectrometry. N.B. carried out flow cytometry experiments and S.M. carried out phenotyping and mouse xenograft experiments both under the supervision of S.H. F.V. contributed luciferase mRNA localization data and were supervised by J.A.C.O. G.-H. contributed computational data analysis. D.S. produced and purified full-length HDLBP. S.D.G. contributed validation experiments. U.Z., M.M., and M.L. interpreted data with input from all authors and wrote the manuscript with contributions from all authors.

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Competing interests
The authors, M.M., C.H.V.-Y., S.M., G.-H., S.D.G., D.S., N.B., F.V., J.A.C., S.K., S.H., and M.S. declare no competing interests. The authors U.Z., I.M., and M.L. declare the following competing interests. U.Z., I.M., and M.L. are co-inventors on a provisional patent application filed by the Max-Delbrück Center for Molecular Medicine related to results presented in Fig. 5.

Additional information
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