Alternative 3’ UTRs act as scaffolds to regulate membrane protein localization

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About half of human genes use alternative cleavage and polyadenylation (ApA) to generate messenger RNA transcripts that differ in the length of their 3’ untranslated regions (3’ UTRs) while producing the same protein1–3. Here we show in human cell lines that alternative 3’ UTRs differentially regulate the localization of membrane proteins. The long 3’ UTR of CD47 enables efficient cell surface expression of CD47 protein, whereas the short 3’ UTR primarily localizes CD47 protein to the endoplasmic reticulum. CD47 protein localization occurs post-translationally and independently of RNA localization. In our model of 3’ UTR-dependent protein localization, the long 3’ UTR of CD47 acts as a scaffold to recruit a protein complex containing the RNA-binding protein HuR (also known as ELAVL1) and SET to the site of translation. This facilitates interaction of SET with the newly translated cytoplasmic domains of CD47 and results in subsequent translocation of CD47 to the plasma membrane via activated RAC1 (ref. 5). We also show that CD47 protein has different functions depending on whether it was generated by the short or long 3’ UTR isoforms. Thus, ApA contributes to the functional diversity of the proteome without changing the amino acid sequence. ApA contributes to the functional diversity of the proteome without changing the amino acid sequence. Thus, ApA contributes to the functional diversity of the proteome without changing the amino acid sequence. ApA contributes to the functional diversity of the proteome without changing the amino acid sequence. ApA contributes to the functional diversity of the proteome without changing the amino acid sequence. ApA contributes to the functional diversity of the proteome without changing the amino acid sequence. ApA contributes to the functional diversity of the proteome without changing the amino acid sequence.

Alternative 3’ UTR isoform abundance was shown to be highly cell-type-specific and can change upon proliferation, differentiation and transformation1–5. Alternative 3’ UTR isoforms produce the same protein, but the long 3’ UTRs contain additional regulatory elements that can regulate mRNA localization and protein abundance6–9. We have discovered a new function of 3’ UTRs: they can regulate protein localization independently of RNA localization.

CD47 is best known as a ubiquitous cell surface molecule that acts as a marker of self and protects cells from phagocytosis by macrophages10,11. We found CD47 protein expressed on the cell surface, as well as intracellularly (Fig. 1a and Extended Data Fig. 1a–c). The CD47 gene produces alternative 3’ UTRs as determined by the 3’-end sequencing method 3’-seq and confirmed by northern blot analysis (Fig. 1b, c). Exclusive knockdown of the longer 3’ UTR isoform by short hairpin RNAs (shRNAs) decreased CD47 surface expression by without changing intracellular expression (Fig. 1d and Extended Data Fig. 1d–g). This suggests that the long 3’ UTR isoform facilitates cell surface localization of CD47 protein.

To test this hypothesis, we asked whether green fluorescent protein (GFP) encoded by an mRNA containing the long (with a mutated proximal polyadenylation signal; Extended Data Fig. 1h) or the short 3’ UTR of CD47 would localize differently. To allow GFP to enter the secretory pathway, we replaced the extracellular domain (ECD) of CD47 with GFP, while preserving the CD47 signal peptide, transmembrane domains (TMDs) and carboxy terminus, which we refer to as GFP-TM (Fig. 1e). We observed that GFP-TM encoded by an mRNA containing the long 3’ UTR of CD47 (GFP-TM-LU) localizes primarily to the cell surface whereas GFP-TM encoded by an mRNA with the short 3’ UTR of CD47 (GFP-TM-SU) localizes predominantly to the endoplasmic reticulum (Fig. 1f and Extended Data Fig. 1i). The localization results were confirmed by fluorescence-activated cell sorting (FACS) analysis, using an anti-GFP antibody on permeabilized and non-permeabilized cells to measure total and surface GFP levels, respectively (Fig. 1g). The localization step occurs at the protein level, as both the LU- and SU-containing GFP transcripts show a similar distribution near the perinuclear endoplasmic reticulum (Fig. 1h). Thus the LU isoform of CD47 encodes information that is necessary for cell surface expression of GFP-TM protein, in a manner independent of RNA localization.

To address the mechanism of 3’ UTR-dependent protein localization (UDPL; Fig. 2a), we reasoned that there must be an RNA-binding protein (RBP) that binds to the long, but not the short, 3’ UTR of CD47. The long 3’ UTR of CD47 contains many uridine-rich elements (see later), which are potentially bound by HuR6–9. HuR is known for its role in mRNA stabilization and translation activation12–14. However, HuR knockdown by shRNAs did not affect CD47 mRNA abundance or isoform levels, nor did it affect total CD47 protein levels (Fig. 2b, bottom, and Extended Data Figs 1d, 2a–c). But, strikingly, knockdown of HuR reduced CD47 surface expression (Fig. 2b, top, and Extended Data Fig. 2c). This suggests that for CD47, HuR mediates protein localization post-translationally.

Beyond the role of HuR as an RBP6–9, HuR interacts through protein–protein interactions with SET, ANP32A and ANP32B4. Nuclear SET binds to histone tails and prevents acetylation15, but phosphorylated SET localizes to the cytoplasm and the surface of the endoplasmic reticulum16. Also, SET interacts with RAC1, and active RAC1 translocates SET to the plasma membrane. In our model of UDPL (Fig. 2a), HuR binds to the long 3’ UTR of CD47 and recruits SET. Upon targeting of the mRNA to the endoplasmic reticulum surface, the scaffold function of the 3’ UTR results in local recruitment of SET to the site of translation. After translation of CD47 mRNA, the ECD is located in the endoplasmic reticulum lumen, whereas its C terminus is cytoplasmic. This allows SET to interact with the newly translated C terminus and cytoplasmic domains of CD47 and to translocate CD47 to the plasma membrane via active RAC1. Transfer of SET from CD47 mRNA to CD47 protein probably requires energy input, as has been shown for transfer of the signal peptide from the signal recognition particle to the translocation channel17. In this model of UDPL, surface expression of CD47-LU depends on SET and active RAC1. And indeed, knockdown of SET or RAC1 by shRNAs reduced surface expression of CD47 without affecting overall CD47 levels (Fig. 2b and Extended Data Fig. 2c–e).

To determine if UDPL is a more widespread phenomenon, we examined the localization of four additional transmembrane proteins that are derived from mRNAs with 3’ UTR isoforms that can be bound by HuR6–9 (Extended Data Figs 3 and 4). TSPAN13 has only one
3′ UTR isoform, whereas the other three genes (CD44, ITGA1 and TNFRSF13C encoding the BAFF receptor, BAFFR) use ApA to generate alternative 3′ UTR isoforms (Fig. 2c and Extended Data Fig. 20). As was the case for CD47, knockdown of HuR decreased surface expression of all four proteins without changing total protein levels (Fig. 2c and Extended Data Fig. 2a, f). As was done for CD47, we generated GFP-fused LU and SU constructs for CD44, ITGA1 and TNFRSF13C with their respective TMDs, C termini and 3′ UTRs. For all tested genes, the longer 3′ UTR increased surface localization of GFP-TM (Figs 1f–h, 2d and Extended Data Fig. 2g). This demonstrates that UDPL has the potential to be a widespread phenomenon.

The 3′ UTR of CD47 contains over 30 putative HuR-binding sites (Extended Data Fig. 3). We tested whether a 3′ UTR with a few HuR-binding sites (HuR-BS) (Extended Data Fig. 3) is enough to mediate surface localization. Indeed, the uridine-rich sequence was necessary and sufficient for surface localization of GFP-TM, although it was less potent than the full-length 3′ UTR of CD47 (Fig. 2c).

Next, we investigated each step of our UDPL model in more detail. We demonstrated by RNA-immunoprecipitation that HuR binds to the HuR-BS and to the LU but not to the SU isoform of CD47 (Fig. 3a, left). SET also associates with the long 3′ UTR of CD47, which is dependent on HuR (Fig. 3a, right). SET or HuR overexpression was insufficient to localize GFP-TM-SU to the cell surface (Extended Data Fig. 5a, b). However, tethering of SET or HuR to the short 3′ UTR isoform of CD47 was sufficient to redirect GFP-TM localization from the endoplasmic reticulum to the plasma membrane (Fig. 3b and Extended Data Fig. 5c, d; see Extended Data Fig. 5 for experimental details). This indicates that local recruitment of SET to the site of translation, mediated by the scaffold function of the long 3′ UTR, is required for UDPL.

Furthermore, by co-immunoprecipitation we demonstrated that endogenous SET only interacts with CD47-LU protein, but not with CD47-SU protein (Fig. 3c). Since SET binds to lysine residues, we mutated single lysines in the C terminus of CD47, which decreased GFP-TM surface localization by up to 57% (Extended Data Fig. 6a). Mutation of 2/5 lysines decreased it by more than 50% and deletion of the entire C terminus decreased it by 80% (GFP-TM-LUΔC; Fig. 3d and Extended Data Fig. 6b). Additional mutation of three lysines in the first cytoplasmic loop abolished surface GFP expression (GFP-TM-LUΔCL; Fig. 3d and Extended Data Fig. 6b). The reduction in surface expression is probably due to partial or complete loss of SET binding to CD47, as demonstrated by co-immunoprecipitation (Fig. 3e).

To test if the difference in surface localization has phenotypic consequences, we added the ECD of CD47 to the GFP constructs (called CD47-LU or CD47-SU; Fig. 4a, f). Both constructs resulted in comparable overall CD47 protein levels (Extended Data Fig. 7a). CD47-LU efficiently localized to the cell surface via UDPL mediated by active RAC1 (Fig. 4b, c and Extended Data Fig. 7b). Whereas GFP expressed from the GFP-TM-SU construct nearly completely localized to the endoplasmic reticulum (Fig. 1f–h), CD47-SU primarily localizes to the endoplasmic reticulum, but also localizes partially to the cell surface, but independently of active RAC1 (Fig. 4b, c).

Figure 1 | The long 3′ UTR of CD47 localizes GFP-TM protein to the plasma membrane, whereas the short 3′ UTR localizes it to the endoplasmic reticulum. a, Fluorescence confocal microscopy of endogenous CD47 protein in non-permeabilized (top) and permeabilized (bottom) cells. IC, intracellular. b, 3′-seq analysis of naïve B cells shows two 3′ UTR isoforms of CD47 mRNA (short 3′ UTR (SU) and long 3′ UTR (LU)). Shown is the last exon of the gene model. Isoform abundance shown in transcripts per million (TPM). c, Northern blot analysis of human cell lines confirming CD47 mRNA isoforms from b. The corresponding ethidium-bromide-stained RNA gel is shown as loading control. d, Staining of U2OS cells as in a after transfection of a control shRNA (sh Co) or an shRNA against the long CD47 3′ UTR isoform. e, CD47 protein contains an N-terminal signal peptide (S; green), ECD (blue), five TMDs (grey) and a cytoplasmic C terminus (C; red). In both constructs, the ECD was replaced with GFP and either fused with the long (GFP-TM-LU) or the short CD47 3′ UTR (GFP-TM-SU). Constructs are drawn to scale. f, Fluorescence confocal microscopy of fixed U2OS cells after transfection of GFP-TM-LU or GFP-TM-SU. Bottom, with additional staining of the endoplasmic reticulum with anti-calnexin. g, FACS analysis of GFP expression in transfected U2OS cells with (dark blue lines, detection of total expression) and without permeabilization (light blue lines, detection of surface (surf.) expression). Values for mean fluorescence intensity (MFI) are shown in parentheses. Unstained cells are shown in grey. Representative image from more than 20 experiments. h, RNA-fluorescence in situ hybridization (FISH) (red) against GFP in permeabilized U2OS cells after transfection of GFP-TM-LU or GFP-TM-SU. Bottom panel also shows GFP-TM protein. a, d, f and h are representative images from hundreds of cells. Scale bars, 10 μm.
Figure 2 | 3′ UTR-dependent protein localization (UDPL) depends on HuR, SET and RAC1, and mediates surface localization of membrane proteins. a, Model of UDPL. HuR binds to the long 3′ UTR and recruits SET. During translation of CD47 mRNA, this protein complex is targeted to the endoplasmic reticulum (ER) surface where SET binds to the newly translated cytoplasmic domains of CD47. This step probably requires energy. SET interacts with RAC1 and active RAC1 translocates SET and CD47 to the plasma membrane. b, FACS analysis of endogenous CD47 protein expression in HEK293 cells. Left panel is analysis of endogenous CD47 protein expression in HEK293 cells. Left panel is shown as in Fig. 1g. Representative images from three (sh2 HuR, n = 5) biological replicates.

We added the respective ECDs to CD44 and BAFFR, which also increased surface expression of their SU isoforms compared with their GFP-TM isoforms, but to a lesser extent than was observed for CD47 (Extended Data Fig. 7c, d). Di- or multimerization of cell surface receptor subunits, which often occurs through their ECDs, is a common strategy for overcoming endoplasmic reticulum retention, because it results in masking of endoplasmic reticulum retention signals.23 We speculate that CD47-SU, CD44-SU and BAFFR-SU might use such a mechanism (although the multimerization partners are unknown) for their partial surface expression. In the case of BAFFR, the ECD only increased surface expression by 1.2-fold, indicating that BAFFR does not co-localize with RAC1 at the plasma membrane. Taken together, our data suggest that membrane proteins rely on UDPL for surface expression to varying degrees.

Cells with high CD47 surface levels are protected from phagocytosis by macrophages (Extended Data Fig. 7e)24. To examine if the difference in surface expression of CD47-LU and CD47-SU protects cells to a different extent from phagocytosis, we used CD47-deficient Jurkat cells (called JinB8 cells25) and expressed similar total amounts of CD47-LU or CD47-SU (Extended Data Fig. 7a). Co-culture of these cells with macrophages demonstrated that CD47-LU fully protected the cells, whereas CD47-SU only partially protected the cells from phagocytosis (Fig. 4d).

CD47 also functions in the regulation of apoptosis26, as JinB8 cells or tissues from Cd47-knockout mice27 fail to undergo apoptosis after γ-irradiation24,25. Interestingly, expression of CD47-SU in JinB8 cells restored apoptosis, but expression of CD47-LU did not affect the loss-of-apoptosis phenotype (Fig. 4e and Extended Data Fig. 7f, g). Thus, when a cell requires increased CD47 surface expression, transcriptional upregulation alone would be non-optimal as it would confer increased susceptibility to apoptosis. ApA-generated 3′ UTR isoforms allow independent regulation of differentially localized and functionally distinct CD47 protein.

As the surface localization of CD47-SU is RAC1-independent, it also does not co-localize with RAC1 at the plasma membrane (Fig. 4f). In contrast, CD47-LU shows strong co-localization with RAC1 at lamellipodia (Fig. 4f). Both activated RAC1 and CD47 are necessary for efficient cell migration and activated RAC1 localizes to the leading edge of migrating cells.5,23,26 We show that only the expression of CD47-LU resulted in changes in cell morphology with the generation of lamellipodia at the leading edge of cells. Furthermore, CD47-LU, but not CD47-SU, resulted in increased active RAC1 (Fig. 4g), which suggests that CD47-LU may cooperate with RAC1 during cell migration. Thus, CD47 protein localized to the same cellular compartment, but produced by the SU or LU mRNA isoforms, can exert different functions. It is currently not known if other surface proteins derived from their LU or SU isoforms also have distinct biological roles.

We propose that UDPL is a widespread mechanism for surface expression of membrane proteins. All currently known components of the pathway (HuR, SET and RAC1) are ubiquitously and highly
Figure 3 | Mechanism of UDPL. a, Left, RNA-immunoprecipitation after transfection of the indicated constructs into HEK293 cells. Protein–RNA complexes were pulled-down with anti-HuR antibody and GFP abundance was normalized to GAPDH and is shown as fraction of input. Right, RNA co-immunoprecipitation after transfection of the indicated shRNAs. Protein–RNA complexes were pulled-down with anti-SET antibody and the abundance of CD47-LU was normalized to GAPDH and is shown as fraction of input. Shown is mean ± standard deviation (s.d.), n = 3 biological replicates. **P < 0.0003, ***P < 0.002, NS, not significant (P > 0.05), two-sided t-test for independent samples. b, MS2-binding sites (see Extended Data Fig. 5) were added to GFP-TM-SU and co-transfected with MS2-mC (left), MS2-mC-HuR (middle) or MS2-mC-SET (right), mC, mCherry. Fluorescence confocal microscopy of HEK293 cells after transfection of indicated constructs shows that recruitment of HuR or SET to the short 3’ UTR redirects localization of GFP protein from the endoplasmic reticulum to the cell surface. Representative images from hundreds of cells. Scale bars, 10 μm. c, Co-immunoprecipitation of endogenous SET using anti-GFP antibody after transfection of CD47-SU or CD47-LU in HEK293 cells (for constructs, see Fig. 4a). Two per cent of input was loaded. WB, western blot. d, FACS analysis of surface GFP expression after transfection of GFP-TM-LU (dark blue line), GFP-TM-LU with a C-terminal deletion (ΔC; light blue line; left) or with destruction of both SET-binding sites (ΔC and K163A, K166A, K175A; ΔCL; light blue line, right). Shown as in Fig. 1g. Representative image from more than four experiments. e, Co-immunoprecipitation of endogenous SET using anti-GFP antibody after transfection of the indicated constructs. Two per cent of input was loaded. Asterisk indicates unspecific band.

Figure 4 | CD47 protein has different functions depending on whether it was generated by the SU or LU isoform. a, To generate GFP–CD47, GFP was inserted in frame between the signal peptide and the rest of the CD47 open reading frame. GFP–CD47 was fused with either the long or short CD47 3’ UTR, called CD47-LU and CD47-SU, respectively. b, FACS analysis of surface (surf.; light blue) and total (dark blue) GFP–CD47 expression in transfected JinB8 cells. Shown as in Fig. 1g. Representative images from four experiments. c, FACS analysis of GFP expression after transfection of CD47-LU or CD47-SU with or without co-transfection of dominant-negative RAC1 (N17RAC1). Shown as in Fig. 1g. Representative images from n = 7 (LU) and n = 2 (SU) experiments. d, Fraction of Mitomycin-C-treated cells that survived at day (d)3 after co-culture with macrophages is displayed for Jurkat, JinB8 (CD47LU) and the GFP+ JinB8 cells after nucleofection of CD47-LU or CD47-SU. Shown is mean ± s.d., n = 3 biological replicates. **P < 0.005, *P < 0.02, NS, not significant (P > 0.05), two-sided t-test for independent samples. e, The fraction of surviving cells (TO-PRO3 negative) measured by FACS analysis at day 3 after γ-irradiation is shown for the same populations as in d. Shown is mean ± s.d., n = 3 biological replicates of the 20% of cells with the highest GFP expression. Gy, Gray. f, Fluorescence confocal microscopy of permeabilized U251 cells after transfection of CD47-LU or CD47-SU co-transfected with anti-RAC1 antibody. Yellow indicates co-localization. Representative images from hundreds of cells. Scale bars, 10 μm. g, Immunoprecipitation of endogenous RAC1–GTP (active RAC1) in HEK293 cells after transfection of CD47-LU, CD47-SU, or empty vector. Total RAC1 and GFP–CD47 were measured from input. n = 3 biological replicates.
expressed (Extended Data Fig. 8a)\(^3\). UDPL requires the presence of HuR-binding sites in the 3′ UTR and SET-binding sites in the cytoplasmic domains of membrane proteins (Extended Data Figs 3, 4 and 9). All candidates tested so far that met both requirements used UDPL for surface expression (Figs 1f–h, 2d, 4b and Extended Data Figs 2g, 7c). HuR-binding sites are highly abundant as HuR binds to thousands of mRNAs\(^8\), with a third of them being membrane proteins (Extended Data Fig. 8b). Although the SET-binding motif is currently unknown, we (Fig. 3d, e and Extended Data Fig. 6) and others have shown that SET binds to positively charged amino acids in histone tails or cytoplasmic domains of membrane proteins\(^9-27\). According to the positive-inside rule for integral membrane proteins, the cytoplasmic domains of membrane proteins are enriched in positively charged amino acids for topological reasons\(^28\). Therefore, potential SET-binding sites in cytoplasmic domains of membrane proteins are very widespread.

So far, efforts to determine the consequences of alternative 3′ UTRs have largely focused on mRNA stability and translation\(^11,29\). We expand the known functions of 3′ UTRs and show that they can act as scaffolds for RBPs that serve as adaptors to recruit effector proteins to the site of translation, which determines subcellular protein localization and function. With respect to CD47, CD44, ITGA1, TSPAN13 and BAFFR, the adaptor protein is HuR and the effector protein is SET. In further studies, we show that scaffold function of 3′ UTRs may extend beyond the regulation of membrane proteins. RBPs could recruit other effector proteins, for example, enzymes that post-translationally modify proteins, as was shown for long non-coding RNAs\(^6\). We showed here that CD47 produced by alternative 3′ UTR isoforms localizes to different cellular compartments and has independent and sometimes opposite functions with respect to cell survival and cell migration. Thus, through the generation of alternative 3′ UTR isoforms, ApA contributes to functional diversity of the proteome without changing the amino acid sequence.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Author Information** Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to C.M. (mayrc@mskcc.org).
METHODS

Cell lines. MCF7 (breast cancer), HeLa (cervical cancer), HEK293 (embryonic kidney), Caov-3 (ovarian carcinoma), NTERA2 (embryonic carcinoma) and THP-1 cells (monocytic leukemia) were purchased from ATCC. B-LCL are Epstein Barr virus (EBV)-immortalized human B cells described elsewhere. U2OS cells (sarcoma) were a gift from T. Brummelkamp, Toledo (B-cell lymphoma) cells were a gift from M. Mueschen, U251 (glioblastoma) cells were a gift from I. Mellinghoff, SHSY-5Y (neuroblastoma) cells were a gift from T. Tuschi and Jurkat (T-cell leukemia) and Jind8 (CD47-negative Jurkat) cells were a gift from W. Frazier.

Constructs. For some of the shRNA knockdown experiments, pSUPERRethropuro was modified by cloning IRES-GFP (derived from pMSCV-pig) downstream of puromycin to obtain pSUPERRethropuro containing enhanced (e)GFP (shRNA-GFP). The following DNA oligonucleotides served as shRNA precursors and were cloned into pSUPER-GFP or pSUPER-CD47annealing two DNA oligonucleotides that were inserted into the BamHI site. To generate the CD47 were cloned from Toledo, SHSY-5Y and B-LCL cDNA respectively and inserted amplified from Toledo cDNA using the TM reverse primer and the CDS forward of (NEB) into pcDNA3.1 vector used above downstream of eGFP. To generate the breast cancer cell line MDA-MB231 and cloned using Gibson Assembly Cloning (NEB) into pcDNA3.1 vector used downstream of eGFP (BsrGII, NotI). The 24 MS2-binding sites were cloned from the plasmid obtained from J. Gerst34 using the primers listed later (XbaI, ApaI). The stop codon was generated by annealing two DNA oligonucleotides that were inserted into the BamHI site. mCherry was PCR-amplified and replaced GFP (BamHI, BsrGI). After cloning was complete, all clones were grown in the presence of high amounts of puromycin (4 mg ml−1) and FACS analysis was performed to ensure that >90% of the cells were GFP+.

Generation of cell lines with stable expression of shRNAs. Stable cell lines were generated as described previously. For surface FACS (in order to detect surface protein expression), cells were incubated with mouse anti-CD44-PerCP-Cy5.5 (BD Biosciences, 561246), mouse anti-CD44-PE (BD Biosciences, 561883), chicken anti-GFP (Abcam, ab13970), mouse anti-ITGA1-PE (BD Biosciences, 555749), mouse anti-BAMBI-PE (BD Biosciences, 555680) or rabbit anti-TSPAN13 (Genetex, GTX212555) in FACS buffer A for 30 min at 4 °C and then washed twice in FACS Buffer A. For detection of GFP and TSPAN13, cells were then incubated with goat anti-chicken Alexa Fluor 568 or 633 (Invitrogen, A11041 or A21103) and goat-anti-rabbit Alexa Fluor 680 (Invitrogen, A-21076), respectively, for 30 min at 4 °C, and then washed twice in FACS Buffer A. At least 30,000 cells were analysed on a BD FACSCalibur cell analyser (BD Biosciences) and FACS data were computed using the FlowJo VX software.

For intracellular FACS (in order to detect total protein expression), cells were fixed in FACS buffer A (0.1% Tween 20 in PBS), permeabilized for 10 min at 4 °C in permeabilization buffer (0.02% sodium azide, 0.1% Tween 20 and 10% dimethyl sulfoxide in PBS), washed, re-fixed for 5 min at room temperature in fixation buffer, and washed again. Cells were incubated with the same primary and secondary antibodies as for surface FACS in FACS buffer B for 30 min at 4 °C, and then washed twice in FACS Buffer B. At least 10,000 cells were analysed on a BD FACSCalibur cell analyser (BD Biosciences) and FACS data were computed using the FlowJo VX software.

FACS analysis. For surface FACS (in order to detect surface protein expression), cells were incubated with mouse anti-CD47-PerCy5.5 (BD Biosciences, 561261), mouse anti-CD44-PE (BD Biosciences, 561883), chicken anti-GFP (Abcam, ab13970), mouse anti-ITGA1-PE (BD Biosciences, 555749), mouse anti-BAMBI-PE (BD Biosciences, 555680) or rabbit anti-TSPAN13 (Genetex, GTX212555) in FACS buffer A for 30 min at 4 °C and then washed twice in FACS Buffer A. For detection of GFP and TSPAN13, cells were then incubated with goat anti-chicken Alexa Fluor 568 or 633 (Invitrogen, A11041 or A21103) and goat-anti-rabbit Alexa Fluor 680 (Invitrogen, A-21076), respectively, for 30 min at 4 °C, and then washed twice in FACS Buffer A. At least 30,000 cells were analysed on a BD FACSCalibur cell analyser (BD Biosciences) and FACS data were computed using the FlowJo VX software.

Immunocytochemistry. For surface staining of CD47, cells were fixed for 15 min at room temperature in fixation buffer A (4% PFA and 0.02% sodium azide in PBS), washed with PBS, blocked for 15 min at 4 °C in 5% Normal Goat Serum

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obtain at least three biological replicates. The following primers were used to
performed in triplicate. The experiments were performed at least three times to
7500 HT Fast Real-Time PCR System (Applied Biosystems). Each reaction was
the manufacturer’s protocol. cDNA was synthesized using random hexamers and
ACAAGTTCAGC-3
Northern blot analysis.

body was incubated for 1 h at 4
a Leica TCS SP5 confocal microscope, using a 63x, 1.4 numerical aperture oil
objective.

For intracellular staining of CD47, cells were fixed for 15 min at room temper-
ature in fixation buffer B (4% PFA, 0.02% sodium azide, and 0.1% TWEEN 20 in PBS), washed in wash buffer B (0.02% sodium azide, 0.1% TWEEN 20 in PBS), permeabilized for 10 min at 4 °C in permeabilization buffer (0.02% sodium azide, 0.1% TWEEN 20 and 10% dimethyl sulfoxide in PBS), washed, re-fixed for 5 min at room temperature in fixation buffer B, washed and blocked for 15 min at 4 °C in 5% Normal Goat Serum in wash buffer. Mouse anti-CD47 (Santa Cruz, sc-90790) primary antibody was incubated overnight at 4 °C in wash buffer. The strong permeabilization and overnight staining were necessary to visualize intracellular CD47, as the CD47 antibody recognizes an epitope in the ECD of CD47, which is
located in the lumen of the endoplasmic reticulum. Owing to the extended treat-
ment with a buffer containing TWEEN 20 the plasma membrane could no longer be
visualized in these cells.

For co-staining of GFP with calnexin or RAC1, the cells were fixed for 15 min at room temperature in fixation buffer B (4% PFA, 0.02% sodium azide, and 0.1% TWEEN 20 in PBS), washed with wash buffer B, blocked for 15 min at 4 °C in 5% Normal Goat Serum (Invitrogen, PC50000) in wash buffer and then incubated with rabbit anti-calnexin (Santa Cruz, sc-11937) or mouse anti-RAC1 (Abcam, ab20248) primary antibodies for 1 h at 4 °C in wash buffer. After washing, goat
anti-rabbit Alexa Fluor 680 (Invitrogen, A-21076) or donkey anti-mouse Alexa Fluor 594 (Invitrogen, A-21203) secondary antibodies were incubated for 1 h at 4 °C in wash buffer, and during the last 10 min of incubation DAPI (Invitrogen, D1306) was added, followed by three washes. Mounting and imaging was performed as for the surface staining. Owing to the lack of permeabilization and short period in TWEEN 20, the plasma membrane was still visible. When calnexin was co-
stained with endogenous CD47, the protocol for intracellular staining of CD47 was
used and the plasma membrane was again not visible.

Alexa Fluor 594 and 680 were pseudo-coloured red and endogenous GFP and mCherry were imaged as they appear, without any antibody.

RNA-FISH. Custom Stellaris FISH Probes (Biosearch Technologies) were designed for the open reading frame of eGFP using the Stellaris Probe designer website, and with the assistance of Biosearch Technologies staff. The probes were conjugated to the Quasar 670 fluorochrome. Staining was carried out according to the manufacturer’s protocols. Briefly, 24 h after transfection of the GFP-TM constructs cells were trypsinized and plated on Millicell EZ glass slides (Millipore) and allowed to grow overnight. Cells were washed in PBS, fixed in 4% PFA at room temperature for 10 min and permeabilized in 70% ethanol at 4 °C for 2 h. After washing, the probes were hybridized at 35 °C for 4 h, washed, incubated with
DAPI at 37 °C for 30 min, washed and mounted in Mowiol mounting media. Imaging was performed as for the surface immunostaining. RNA was pseudo-
coloured red, and GFP was imaged as it appears, without any antibody.

Northern blot analysis. Northern blots were performed as previously described2. The primers used were as
earlier, but using anti-SET (Abcam, ab181990) or IgG (Santa Cruz, sc-2025). The
earlier, but using anti-SET (Abcam, ab181990) or IgG (Santa Cruz, sc-2025). The
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earlier, but using anti-SET (Abcam, ab181990) or IgG (Santa Cruz, sc-2025). The
earlier, but using anti-SET (Abcam, ab181990) or IgG (Santa Cruz, sc-2025). The
cDNA was synthesized using random hexamers and the TaqMan Reverse Transcription Kit (Applied Biosystems). qRT–PCR was performed using the Power SYBR Green master mix (Applied Biosystems) on a 7500 HT Fast Real-Time PCR System (Applied Biosystems). Each reaction was performed in triplicate. The experiments were performed at least three times to obtain at least three biological replicates. The following primers were used to quantify total CD47 mRNA, the long 3' UTR isoform of CD47 and GAPDH for normalization.

CD47TotalF: 5'-AGTGATGAGCTCCAGTGGT-3'; CD47TotalR: 5'-GGC
TCTCATAGGGACACACGACG-3'; CD47LongF: 5'-AGAGAAGCTCGAGATG-3'
GCT-3'; CD47LongR: 5'-AGCTGAAGACAGCTGAAAACA-3'; GAPDH:
5'-ACAACTTGGATATCGTGGAAG-3'; GAPDHr: 5'-TATTGGCAGCTT
TTCTAGAAG-3'.

To measure 3' UTR isoform expression by qRT–PCR and to take into account different affinities of primers, we generated a standard using plasmas that
contained either the short or the long 3' UTR of CD47 (see earlier). We mixed

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an Odyssey CLx imaging system (Li-Cor). Quantification of western blots was performed using Image J.

**Co-immunoprecipitation.** CD47-SU, CD47-LU, CD47-LU/CL and CD47-LU/AC were transfected into HEK293 cells and the cells were lysed in ice cold RIPA buffer (125 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% Na-deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitor cocktail (Roche, 04693124001)) for 5 min on ice. After the cells were spun down at 20,000 g for 20 min the supernatant was pre-cleared as described earlier. The lysate was divided in two equal parts (a small portion was removed to be used as the input control). Anti-GFP (Invitrogen, A-6455) or IgG (Santa Cruz, sc-2025) was added to the lysates and a 1 h rocking incubation at 4 °C was performed, followed by addition of magnetic protein A beads (Millipore, LSKMAGA10) and another 1 h rocking incubation at 4 °C. After seven washes in RIPA buffer the beads were then boiled in Laemmli buffer (Sigma, S3401) for 7 min and then cooled on ice. Western blotting was carried out as described earlier. Chicken anti-GFP (Abcam, ab13970) antibody was used to confirm immunoprecipitation of GFP constructs and rabbit anti-SET (Abcam, ab181990) antibody was used to assess co-immunoprecipitation of SET protein.

**Immunoprecipitation of RAC1–GTP.** CD47-SU, CD47-LU and pcDNA 3.1 vector alone were used as an Active Rac1 Detection Kit (Cell Signaling Technology, 8815) following the manufacturer’s protocol. Briefly, cells were lysed in ice cold lysis buffer and centrifuged at 16,000g for 15 min at 4 °C. The supernatant was added to GST-PAK1-PBD and glutathione resin and incubated with rocking for 1 h at 4 °C (a small portion was removed to be used as the input control). The resin was washed three times with lysis buffer and then SDS sample buffer was added to elute the bound proteins. Western blotting was carried out as described earlier. Mouse anti-RAC1 (Cell signaling Technology, 8631S) antibody was used to detect RAC1–GTP as well as to assess total RAC1 in the samples.

**Irradiation and cell survival assay.** Jurkat, JinB8, and transfected JinB8 cells (24 h post-transfection) were irradiated for a total of 0, 5, 10, 20 or 40 Gy using a Shepherd Mark-1 caesium irradiator. For JinB8 or Jurkat cells transfected with CD47-SU or with sh2 CD47-LU the percentage of GFP+ cells was determined by FACS before irradiation. Three days after γ-irradiation, cells were stained with TO-PRO3 (Sloan Kettering Institute, Flow Cytometry Core Facility) as the TO-PRO3-negative cells divided by the total number of GFP+ are mean and standard deviation of three biological replicates.

**Phagocytosis assay.** Human macrophages were obtained by differentiation of THP-1 cells with 25 ng ml−1 phorbol 12-myristate 13-acetate (PMA; Sigma) for 3 days. On day 3, Jurkat, JinB8, or transfected JinB8 cells were treated with 10 μg ml−1 Mitomycin C for 2.5 h at 37 °C and washed for three times in media. Mitomycin C treatment halts cell division and allows for a more accurate assessment of the percentage of cells that are phagocytosed. For JinB8 cells transfected with CD47-LU or CD47-SU the percentage of GFP+ cells was determined by FACS before co-culture. The cells were either cultured alone or co-cultured with fully differentiated macrophages. After 3 days, cells were counted and the fraction of GFP+ cells was determined by FACS analysis. The fraction of surviving cells after co-culture with macrophages was normalized by the number of surviving cells without co-culture and is show as mean and standard deviation of three independent experiments.

To demonstrate that the cells were phagocytosed, during the last 10 min of Mitomycin C treatment the cells were also labelled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen). Washing after Mitomycin C and CFSE treatment was carried out in cold media according to the manufacturer’s protocol. After co-culture, CFSE uptake by macrophages was measured by FACS analysis to demonstrate that a decrease in the number of surviving cells is due to phagocytosis by macrophages.

**Fraction of membrane proteins among HuR target genes.** The list of HuR target genes was obtained from previous publications31. The union of genes from both publications was analysed using gene ontology analysis32 and all genes with the tag “membrane” were considered membrane proteins. This number is consistent with the number of membrane proteins obtained in yeast33. Fisher’s exact test was used to test for significance.

**Statistical analysis.** To test for significant differences between samples a two-sided t-test for independent samples was performed using SPSS.
Extended Data Figure 1 | Expression of the long CD47 3’ UTR isoform correlates with cell surface expression of CD47 protein. a, Fluorescence confocal microscopy of cells shown as in Fig. 1a. Representative images out of hundreds of cells are shown. Scale bars, 10 μm. b, FACS analysis of endogenous CD47 expression in cells shown in Fig. 1a and a. Permeabilized cells show total CD47 expression (purple) and non-permeabilized cells show surface CD47 expression (blue). Representative histograms are shown (HEK293 cells, n = 10; U2OS, Jurkat cells, n = 5; Caov-3, n = 3). Unstained cells are shown in grey. c, Left, quantification of mean fluorescence intensity (MFI) values from b. Right, fraction of surface and intracellular CD47 levels in cells lines from b. Intracellular CD47 was calculated by subtracting CD47 surface values from total CD47 values. d, Northern blot of HEK293 cells stably expressing the indicated shRNAs and hybridized for CD47. The shRNAs against CD47-LU target only the long 3’ UTR isoforms of CD47. The blot and corresponding RNA gel are shown as in Fig. 1c. e, Quantification of CD47 total mRNA and 3’ UTR isoform levels in U2OS cells by qRT–PCR. GAPDH-normalized values after transfection of sh2 CD47-LU or sh Co are shown as the mean ± s.d., n = 3 biological replicates. The total amount of CD47 mRNA after transfection of sh Co was set to 1. f, FACS analysis of endogenous CD47 protein expression after stable expression of shRNAs against CD47-LU in HEK293 cells. Surface (top) and total (bottom) CD47 expression is shown. Representative histograms out of n = 3 experiments are shown. Unstained cells are shown in grey. g, Quantification of MFI values from f is displayed. Intracellular CD47 was calculated as in b. h, Northern blot of HEK293 cells after transfection of indicated constructs and hybridized against CD47. Mutation of the proximal polyadenylation signal in CD47-LU abrogates production of short 3’ UTR isoforms. Asterisk indicates cross-hybridization to ribosomal RNAs. i, Fluorescence confocal microscopy of endogenous CD47 and calnexin protein in permeabilized U2OS cells. Calnexin partially co-localizes with CD47. A representative image out of hundreds of cells is shown. Scale bars, 10 μm.
Extended Data Figure 2 | UDPL depends on HuR, SET and RAC1 and mediates surface localization of membrane proteins. a, Western blot of HEK293 cells transiently transfected (left, middle) or stably expressing (right) sh Co or shRNAs against HuR. The blot shows reduced HuR protein expression after HuR knockdown, but no change in protein expression of CD47, TSPAN13, CD44 or SET. Actin was used as loading control. b, Quantification of CD47 total mRNA and 3′ UTR isoform levels in HEK293 cells by qRT–PCR. GAPDH-normalized values after transfection of sh2 HuR or sh Co are shown. Shown is the mean ± s.d., n = 3 biological replicates. The total amount of CD47 mRNA after transfection of sh Co was set to 1. c, FACS analysis of HEK293 cells stably expressing the indicated shRNAs. Histograms are shown as in Fig. 2b. Representative histograms from n = 3 experiments are shown. d, Western blot of HEK293 cells stably expressing shRNAs against SET. Actin was used as loading control. The marker is shown in kDa. e, As in d, but HEK293 cells stably expressing shRNAs against RAC1 are shown. f, 3′-seq analysis shows 3′ UTR isoform expression of ITGA1 in B-LCL and TSPAN13 in HEK293 cells shown as in Fig. 1b. FACS analysis of endogenous protein levels is shown as in Fig. 2c. Left panel shows ITGA1 expression in HeLa cells and right panel shows TSPAN13 expression in HEK293 cells. Representative histograms from n = 2 experiments are shown. g, FACS analysis of GFP after transfection of constructs containing a signal peptide and GFP fused to the TMD, C terminus and either the long 3′ UTR (dark blue line) or the short 3′ UTR (light blue line) of ITGA1 in HEK293 cells. Representative histograms from n = 3 experiments are shown as in Fig. 2d.
Extended Data Figure 3 | 3′ UTR isoforms that encode proteins using UDPL contain uridine-rich elements. Shown are the 3′ UTR sequences of CD47, CD44, HuR-BS and HuR-BSA. Red, ApA signals. Blue, uridine-rich elements with the potential to be HuR-binding sites.
Extended Data Figure 4 | 3’ UTR isoforms that encode proteins using UTR sequences of ITGA1, TNFRSF13C and TSPAN13. Red, ApA signals. Blue, uridine-rich elements with the potential to be HuR-binding sites.

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Extended Data Figure 5 | Local recruitment of SET to the site of translation is required for UDPL. a, Western blot of cells used in Fig. 3b shows the amount of overexpression achieved by transfection of MS2-mC-SET or MS2-mC-HuR (for constructs, see b). Left, anti-SET detects endogenous expression of SET as well as overexpressed SET. Right, anti-HuR detects endogenous HuR and overexpressed HuR. Actin was used as loading control. Anti-HuR and anti-SET were used on the same blot. Actin as loading control was performed once. The marker is shown in kDa. Asterisk indicates unspecific band. mC, mCherry. b, The top construct depicts GFP-TM-SU (Fig. 1e) and the bottom construct shows a fusion of MS2 coat protein (MS2), mC (red) and HuR or SET, respectively. Overexpression of HuR or SET compared with expression of MS2-mC alone does not change surface or total GFP expression, when co-transfected with GFP-TM-SU (without the addition of MS2-binding sites to the SU isoform) as shown by FACS analysis. Surface expression (top) and total expression (bottom) in HEK293 cells are shown. Values for MFI are shown in parentheses. Unstained cells are shown in grey. Representative histograms from n = 5 experiments are shown. c, FACS analysis of cells used in Fig. 3b. MS2-binding sites (MS2-BS, RNA stem loops) were added to GFP-TM-SU (c), MS2-binding sites are derived from the bacteriophage MS2 and form RNA stem loops. The capsid protein of MS2 (here, called MS2) specifically recognizes these MS2 stem loops. Constructs were generated containing MS2 fused to mC and then either HuR or SET, respectively. Overexpression of HuR or SET compared with expression of MS2-mC alone does not change surface or total GFP expression, when co-transfected with GFP-TM-SU (without the addition of MS2-binding sites to the SU isoform) as shown by FACS analysis. Surface expression (top) and total expression (bottom) in HEK293 cells are shown. Values for MFI are shown in parentheses. Unstained cells are shown in grey. Representative histograms from n = 5 experiments are shown. d, A si n c, but tethering was impaired by omission of the MS2 coat protein. Histograms are shown as in b. Representative histograms from n = 5 experiments are shown. Summary of the tethering experiment: To tether SET or HuR to the 3’ UTR (which brings it close to the site of translation through the scaffold function of the 3’ UTR), we added MS2-binding sites to GFP-TM-SU (c). Thus, tethering of HuR or SET to the short 3’ UTR of GFP-TM localizes GFP to the cell surface without changing total GFP expression. Histograms are shown as in b. Representative histograms from n = 5 experiments are shown. Summary of the tethering experiment: To tether SET or HuR to the 3’ UTR (which brings it close to the site of translation through the scaffold function of the 3’ UTR), we added MS2-binding sites to GFP-TM-SU (c). The cells that express MS2 fused to only mC localize GFP to the endoplasmic reticulum, but constructs containing MS2 fusions to HuR or SET localize GFP primarily to the cell surface (Fig. 3b and Extended Data Fig. 5c). Omitting either MS2 or the MS2-binding sites from the experiment abrogates surface localization (Extended Data Fig. 5b, d).
Extended Data Figure 6 | CD47 contains at least two SET-binding sites in its cytoplasmic domains. a, FACS analysis of surface GFP expression after transfection of GFP-TM-LU (dark blue line) and GFP-TM-LU constructs containing a single point mutation in the cytoplasmic C terminus (light blue line), K290A (left), K297A (middle), K304A (right) in HEK293 cells. Partial destruction of a single SET-binding site results in up to 37% reduction in GFP surface expression. Values for MFI are shown in parentheses. Unstained cells are shown in grey. Representative histograms from \( n = 5 \) experiments are shown. 

b, FACS analysis of GFP expression after transfection of GFP-TM-LU (dark blue line), GFP-TM-LU containing a mutation of the SET-binding site in the C terminus (K290A, K304A; 2Km; light blue line; left), containing a deletion of the C terminus (ΔC; light blue line; middle panel), or destruction of both SET-binding sites (ΔC combined with K163A, K166A, K175A; ΔCL; light blue line; right). Surface (top) and total (bottom) expression is shown in HEK293 cells. Values for MFI are shown in parentheses. Unstained cells are shown in grey. Representative histograms from several experiments are shown (2Km, \( n = 3 \); ΔC, \( n = 10 \); ΔCL, \( n = 4 \)).
Extended Data Figure 7 | CD47 protein has different functions depending on whether it was generated by the SU or LU isoform. a, Left, western blot of HEK293 cells after transfection of the indicated constructs shows GFP–CD47 expression using an anti-GFP antibody. Actin was used as loading control. Right, as in left panel after transfection of CD47-SU and CD47-LU into HEK293 (left) or JinB8 cells (right). GFP–CD47 expression was quantified after normalization with respect to actin using Image J. Shown is the fold change in GFP–CD47 expression of CD47-SU after setting CD47-LU to 1. b, The experiment is similar to Fig. 3b and Extended Data Fig. 5c, but here the constructs containing the full open reading frame of CD47 were used. FACS analysis of GFP expression after transfection of CD47-SU-MS2-BS. Co-transfection of MS2-mC-HuR (left, dark purple line) or MS2-mC-SET (right, dark purple line) increases surface GFP expression compared to co-transfection of MS2-mC (light purple line). Surface expression is shown in non-permeabilized HEK293 cells. Values for MFI are shown in parentheses. Representative histograms from n = 3 experiments are shown. Unstained cells are shown in grey. c, Left, FACS analysis of GFP after transfection of constructs containing a signal peptide and GFP fused to the open reading frame of BAFFR and either the long 3’ UTR (BAFFR-LU, dark blue line) or the short 3’ UTR (BAFFR-SU, light blue line) in HEK293 cells. Surface (top) and total (bottom) GFP expression is shown. Values for MFI are shown in parentheses. Representative histograms from n = 3 experiments are shown. Unstained cells are shown in grey. Right, as in left panel but for CD44. d, Table showing the fold increase in surface GFP expression mediated by the LU isoform compared with the SU isoform. Top row shows values of constructs without the ECD and bottom row shows values of constructs containing the full coding regions of the indicated proteins. The fold increase in surface GFP expression was calculated from MFI (LU)/MFI (SU). The contribution of the ECD domain for surface expression of BAFFR is 1.2-fold (3.8/3.1). e, FACS analysis of carboxyfluorescein succinimidyl ester (CFSE) uptake in macrophages. Macrophages were co-cultured without (grey) cells or with cells that were pre-treated with CFSE and expressed high or low amounts of surface CD47 (data not shown). The experiment shows that the macrophages phagocytose the cells depending on their CD47 surface expression levels. A representative histogram from n = 2 experiments is shown. f, The fraction of surviving cells (TO-PRO3 negative) as measured by FACS analysis at day 3 (d3) after increasing doses of γ-irradiation is shown for Jurkat, JinB8 (CD47−/−) and the GFP+ fraction after nucleofection of JinB8 cells with either CD47-SU or CD47-LU. The values were obtained from the same experiment as shown in Fig. 4e, but here the values were calculated using all GFP-positive cells. Shown are the values for mean ± s.d., n = 3 biological replicates. Gy, Gray. g, The fraction of surviving cells (TO-PRO3 negative) as measured by FACS analysis at day 3 (d3) after increasing doses of γ-irradiation is shown for Jurkat, JinB8 (CD47−/−) and the GFP+ fraction after nucleofection of Jurkat cells with sh2 CD47-LU. Shown are the 20% of cells with the highest GFP expression (green). Shown are the values for mean ± s.d., n = 3 biological replicates. Gy, Gray.
Extended Data Figure 8 | HuR, SET and RAC1 are widely and highly expressed. a. The mRNAs of proteins necessary for UDPL are ubiquitously and highly expressed across cell lines (left) and tissues (right). Shown are values for transcripts per million (TPM). The median abundance levels of all expressed genes in the data sets are shown as dashed lines. *ELAVL1* encodes HuR. The data set from ref. 3 was analysed to obtain the TPM values. b, Here, ‘HuR targets’ consist of the union of HuR targets identified previously⁷,⁹. Membrane proteins consist of all the proteins that contain the tag “membrane” using gene ontology analysis. The fraction of membrane proteins found is consistent with the fraction of membrane proteins found in yeast⁴⁰. Fisher’s exact test shows no enrichment or depletion of membrane proteins among the HuR targets.
Extended Data Figure 9| All tested UDPL candidates have potential SET-binding sites in their cytoplasmic domains. Shown are the amino acid sequences of the TMDs and cytoplasmic domains of the membrane proteins studied. The TMDs are shown in green and the positively charged amino acids in the cytoplasmic domains, indicating potential SET-binding sites, are shown in red.