Cellular therapies remain constrained by the limited availability of sensors for disease markers. Here we present an integrated target-to-receptor platform for constructing a customizable advanced modular bispecific extracellular receptor (AMBER) that combines our generalized extracellular molecule sensor (GEMS) system with a high-throughput platform for generating designed ankyrin repeat proteins (DARPins). For proof of concept, we chose human fibrin degradation products (FDPs) as markers with high clinical relevance and screened a DARPin library for FDP binders. We built AMBERs equipped with 19 different DARPin selected from 160 hits, and found 4 of them to be functional as heterodimers with a known single-chain variable fragments binder. Tandem receptors consisting of combinations of the validated DARPin are also functional. We demonstrate applications of these AMBER receptors in vitro and in vivo by constructing designer cell lines that detect pathological concentrations of FDPs and respond with the production of a reporter and a therapeutic anti-thrombotic protein.
Fig. 1 | AMBERs are generated using ribosome display and can function in different configurations to detect soluble targets such as GFP or MBP. 

a, Activation of the sensor triggers the signal transducer and activator of transcription 3 (STAT3) transcription factor pathway, which is rerouted towards expression of a therapeutic output (TNK or hirudin, Hir) or a reporter protein (SEAP or NLuc). A four-step pipeline for the generation of functional AMBERs. First, binders (DARPins) are selected by ribosome display and the genetic information of candidate binders is analyzed. Next, genetically distinct binders are used to build AMBERs, which are tested in cellular assays in homodimeric or heterodimeric configuration. AMBERs on the cell surface can be employed to detect fibrin-derived proteins via engineered extracellular binding domains. After the identification of functional binders, they can be used to build more compact tandem receptors with similar specificity. b–d, Spatial requirements of AMBERs were tested by inducing AMBERs specific for GFP on the basis of a published clamp-DARPin (b), a corresponding single DARPin (c) and a nanobody in a homodimeric receptor configuration (d). GFP–GFP fusion proteins having a long GGGGS4 (20 amino acids), short GGGGS1 (5 amino acids) or no linker between the GFP domains were used as analytes. e, An additional AMBER was designed for the detection of bacterial MBP. Its EC50 value was estimated to be 0.85 nM. f, Results from HTRF assay depicted as the ratio of maximum fluorescence of the sample versus the streptavidin control. Streptavidin is used to couple biotinylated target protein to beads during the selection run. DARPins are labeled via a FLAG tag with an antibody coupled to a fluorescent acceptor dye (d2) while xFDPs are labeled in a similar fashion via their biotin tag with streptavidin carrying a Tb FRET donor. Binding of a DARPin to FDP protein hence results in an increase of FRET efficiency (increased signal-to-noise ratio). AU, arbitrary units. EC50 values of clamp-DARPin-based AMBER (b) were 1.14 nM (long linker), 0.77 nM (short linker), and 0.84 nM (no linker) with 95% confidence intervals (CIs) of [0.91 to 1.46], [0.52 to 1.09], and [0.73 to 0.96]; the single DARPin-based AMBER (c) showed EC50 values of 2.27 nM (long linker), 2.00 nM (short linker), and 1.83 nM (no linker) with 95% CIs [1.83 to 2.77], [1.64 to 2.44], and [1.48 to 2.27]; and the nanobody-based AMBER (d) showed EC50 values of 0.49 nM (long linker), 0.34 nM (short linker), and 0.35 nM (no linker) with 95% CIs [0.38 to 0.63], [0.29 to 0.43], and [0.30 to 0.41]. Data are presented as mean ± s.d. of n = 3 independent samples.
by using reported binders to build an AMBER for the detection of bacterial maltose-binding protein (MBP). Then, we characterized the full AMBER pipeline in more detail by generating binders for human fibrin degradation products (FDPs) to build AMBERs that would serve as cellular sensors for blood coagulation events. FDPs are already used as a biomarker to rule out life-threatening pathologic coagulation events\(^{14,15}\). We also demonstrated the translational potential of the AMBER system by engineering mammalian cells equipped with AMBERs to detect pathologoical coagulation events and to respond by triggering the secretion of a therapeutic protein, either tenecteplase (TNK) or the anti-coagulant hirudin.

Overall, our results suggest that the AMBER pipeline provides streamlined and predictable methodology for the generation of new cellular receptors for soluble targets of choice. We believe this technology will facilitate research on cellular signaling, as well as the development of future cellular therapies to treat diseases for which no relevant cellular receptors are yet known.

**Results**

**Design requirements of AMBERs.** We combined the AMBER system (Fig. 1a) with established high-throughput DARPin selection technology to design a four-step pipeline for generating functional AMBERs (Fig. 1a). In brief, DARPinS are selected by ribosome display and their sequences are classified by phylogenetic comparison and pairwise amino acid alignments. Distinct DARPinS are fused to the receptor scaffold and tested for induction in a homodimeric or heterodimeric receptor setup. Finally, we developed a more compact tandem receptor design featuring two binders on a single receptor, thereby reducing the amount of genetic material required for transfection. This design may be applied to validated DARPin binders.

In the AMBER platform, receptor activation relies on binding of both receptor chains to their target, inducing receptor dimerization. From the crystal structure of activated EpoR (Protein Data Bank (PDB) ID: 1EER), we estimated the optimal distance between the attachment sites of the C termini of the two binders (N termini of the EpoR chains; orange in Fig. 1a) to the extracellular domains (green in Fig. 1a) to be less than 8 nm. However, larger structures may also be recognized\(^{22}\).

To characterize the spatial requirements of the receptor system and to benchmark the performance of DARPinS in this context, we built AMBERs equipped with either a clamp-DARPin, consisting of two DARPinS forming a high-affinity binder\(^{16}\), a single DARPin derived from that clamp-DARPin binder\(^{17}\) or a nanobody binder\(^{18}\), each targeting GFP. We transfected HEK-293T cells with these receptors in a homodimeric configuration and induced them with purified GFP–GFP fusion proteins having a long and flexible GGGGS\(_4\) linker, a short GGGGS\(_1\) linker, or no linker at all to allow for differently spaced epitopes (Fig. 1b–d, Supplementary Fig. 1a). A clear effect of linker length on EC\(_{50}\) was observed for clamp-DARPin-based receptors (Fig. 1b–d), Supplementary Fig. 1a and Supplementary Table 3). Nanomolar half maximal effective concentration (EC\(_{50}\)) values were obtained, and GFP–GFP without any linker showed the strongest activation (Fig. 1b–d). A clear effect of linker length on EC\(_{50}\) was observed for clamp-DARPin-based AMBER and nanobody-based AMBER. We also confirmed the functionality of AMBER with scFv/DARPin binder combinations by designing and testing a receptor for bacterial MBP, using a published scFv sequence (PDB ID: 7JTR chain B) together with a DARPin (PDB ID: 1SVX) fused through a stiff (EAAAK)\(_4\) linker, its estimated EC\(_{50}\) was 0.85 nM (Fig. 1c).

To evaluate the AMBER platform for detection of disease markers, we next established functional receptors for D-dimers, which contain identical epitopes along the whole 45-nm length of the dimeric fibrinogen molecule\(^{21,22}\), providing a range of differently spaced epitope pairs.

**DARPin selection.** Multiple intermediates and derivatives of fibrin, which partially share the same epitopes, are formed during coagulation and fibrinolysis (Extended Data Fig. 1). To validate the AMBER platform we chose commercially available cross-linked fibrin degradation products (xFDPs) enriched in D-dimers (fibrin D-fragments linked by an isopeptide bond, compare Extended Data Fig. 1) as a target for FDP binder selection because of their clinical relevance\(^{16}\). This preparation also contains traces of D-E complex (120kDa) and DD-E complex (220kDa) with large surface areas that should present numerous epitopes (Supplementary Fig. 1b).

We biotinylated the xFDP preparation and applied four rounds of in vitro ribosome display-based screening to generate DARPinS (Methods; Extended Data Fig. 2). We then screened 380 DARPinS for target binding by homogeneous time-resolved fluorescence (HTRF)-based assay (Fig. 1f). We verified the absence of unspecific binding to human serum albumin (hSA), present as a stabilizer in the xFDP preparation.

Of the 380 DARPinS, 160 (42%) gave a fold induction >1.15 (a value chosen on the basis of our previous screening experience), and of these, 118 (31%) were considered good binders with a fold induction >1.45. To cover a broad range of epitopes, we selected 24 high-affinity and 8 medium-affinity DARPinS for Sanger sequencing. Among them, 19 DARPinS had unique sequences, though some showed high sequence similarity (Supplementary Fig. 2), and 15 (79%) were monomeric (Supplementary Fig. 3). We used all 19 binders in subsequent receptor designs, as constraints introduced by receptor fusion might affect dimerization in the final receptor constructs.

**Homodimeric, heterodimeric, and tandem AMBER configurations.** Different receptor configurations were explored to find the best setup (Fig. 2a). A homodimeric receptor configuration in which all receptors have the same binding module is the smallest and most straightforward receptor architecture (Fig. 2a). However, homodimeric receptors rely on the presence of two identical epitopes close together, so functional receptors require dimeric or symmetric ligands.

Heterodimeric receptors are pairwise combinations of receptors with different binding moieties (Fig. 2a), and thus require two independent receptor chains. Pairwise combinations grow exponentially with the number of available binders (N pairwise combinations of two out of n elements: N = 0.5n\(^2\) – 0.5n), greatly increasing the likelihood of finding active receptors.

Tandem receptors consist of two binders fused in sequence to the N terminus of the receptor scaffold, resulting in increased avidity and possibly higher sensitivity (Fig. 2a). They require a smaller genetic payload than heterodimeric receptors (2.5–2.9 kb for the receptor alone), allowing them to be used in size-restricted vectors, such as recombinant adeno-associated viruses (rAAVs)\(^{18}\). However, the combinatory space when all binders are tested in all possible configurations in heterodimeric tandem receptors increases to the power of four for each binder.

For AMBER, we therefore decided to screen homodimeric or heterodimeric receptors before confirming the functionality of tandem receptors built with the validated binders.

**AMBER screening setup and specificity controls.** To benchmark new FDP-binding DARPinS and to explore cross-platform binder combinations, we used a single-chain variable fragment (scFv) targeting human D-dimers\(^{24}\). We equipped the AMBER platform with the anti-D-dimer-scFv (scFv-AMBER) and the 19 FDP-binding DARPinS, obtaining 20 receptors, each bearing a single binding moiety on the extracellular domain. HEK-293T cells were co-transfected with plasmids, each encoding a single receptor under the control of a constitutive synthetic promoter derived from human cytomegalovirus (hCMV) (P\(_{hCMV}\)-receptor-pA), along with a plasmid encoding a constitutively expressed STAT3 transcription factor (pLS15, P\(_{hCMV}\)-STAT3-pA) and a secreted human placentald
alkaline phosphatase (SEAP) reporter plasmid controlled by two STAT3 response elements (RE) and a minimal version of PhCMV (pLS13, RE2-PhCMVmin-SEAP-pA).

Insensitivity of the receptors to erythropoietin (EPO) was confirmed by the absence of EPO-mediated activation (Supplementary Fig. 4a). A construct based on an scFv responding exclusively to the non-toxic industrial dye Reactive Red (RR120) was used as a negative control for testing potential receptor-independent effects (Supplementary Fig. 4b). We assessed the impact of purified human xFDPs on the productivity and viability of HEK-293T cells by evaluating constitutive SEAP expression (Supplementary Fig. 4c) and by resazurin-based assay (Supplementary Fig. 4d), respectively. xFDPs were non-toxic up to 10 µg ml⁻¹. Human plasma at concentrations up to 2% (v/v) had little effect on constitutive SEAP expression (Supplementary Fig. 4e) or viability (Supplementary Fig. 4f).

**AMBER screening.** We finally screened all 210 possible pairwise combinations of receptors (including all 20 homodimers) (Extended Data Fig. 3 and Supplementary Fig. 5). Induction with xFDPs yielded four weak (2 < fold induction < 5; 1.9%) and three strong (fold induction > 5; 1.4%) receptors (Fig. 2b). Notably, all receptors responding strongly to xFDPs were heterodimers formed by co-expression of a DARPin with the scFv receptor, indicating the importance of synergies among binders covering different epitopes for the generation of functional receptors.

Human citrate-treated plasma was tested to evaluate the system performance in a more native setting and to investigate responses to FDP species underrepresented in the DARPin selection and screen. We found weak responses for 37 (17.6%) and strong responses for 49 (23%) receptor combinations of which 77% included DARPin A6, A7, B4 or F6. We found no correlation of HTRF signal intensity from DARPin selection with the number of functional receptors or the receptor signal strength in a homodimeric setup (Supplementary Fig. 6). This was not unexpected, since HTRF signal strength depends on both affinity and epitope location. Interestingly, receptor activity in a homodimeric setting seems predictive for the number of functional receptor combinations with fold induction > 2. Some functional receptors contained DARPins that showed oligomerization behavior (for example, F9-DARPin; Supplementary Fig. 3), indicating that auto-dimerization of the binder alone does not necessarily limit its use in a receptor context.

**Characterization of candidate AMBERs.** We next characterized DARPins A6, A7, B4, and less-active DARPin G1, as well as the scFv, in a homodimeric receptor configuration. Reporter levels showed little xFDP concentration dependence for receptors based...
on A6, A7 and B4 DARPin receptors (Fig. 3a). A possible explanation is binding of large antigens to both receptor chains, thereby reducing basal (‘leaky’) activity by stabilizing pre-formed receptor dimers in an inactive conformation. Alternatively, the epitopes in a single xFDP molecule might be oriented such that they cannot be simultaneously engaged by both binders.

By contrast, we recorded ON-type switching behavior with up to 110-fold induction in response to human plasma for the same DARPin receptors, but not for the scFv (Fig. 3d). This may indicate that these DARPin receptors are activated by another protein that is not present in the xFDP preparation (compare Supplementary Fig. 7a,b). Plasma contains fibrinogen, a precursor of xFDPs that is activated by thrombin contained in the medium’s fetal calf serum (FCS) supplement, leading to multiple possible fibrinogen-derived xFDPs (Extended Data Fig. 1). We performed further experiments to identify the responsible molecule (see below). Here, we first focus on the receptor configurations and binder characteristics.

We examined the four candidate DARPin receptors in combination with the scFv as heterodimeric receptor pairs. In contrast to the homodimeric configuration, these receptors dose-dependently respond to xFDPs with inductions of up to 25-fold and EC_{50} values of 0.15, 0.19, and 0.12 μg ml^{-1} for DARPin receptors A6, A7, and B4, respectively (Fig. 3b). These EC_{50} values are below the pathological threshold for D-dimer of 0.5 μg ml^{-1} in blood[41]. Induction with plasma yielded similar dose–response relationships to those in the homodimeric configuration (Fig. 3c). However, the heterodimeric setup also allows for homodimeric receptors at the cell surface, and the latter most likely dominate the plasma induction response. Nevertheless, both heterodimeric receptors were also activated by xFDPs. Thus, it might be possible to distinguish different stages or states of coagulation by using different receptors (compare Extended Data Fig. 1).

**Generating tandem AMBERs.** After identifying functional DARPin binders and scFv combinations in the homodimeric and heterodimeric screens, we fused all 16 combinations of tested DARPin and scFv receptors to the receptor scaffold in tandem orientation (Fig. 2a). Three (19%) receptors, all containing a DARPin in combination with the scFv, were sensitive to xFDPs (Supplementary Fig. 7f). All but one of the DARPin-based receptors and all but two of the scFv–DARPin combinations were plasma responsive. Notably, we found a working receptor for each DARPin successfully tested before (Supplementary Fig. 7a,b). The scFv alone did not respond to either plasma or xFDPs in tandem configuration, in accordance with the results for the homodimeric receptors.

The DARPin-only tandem receptors detected xFDPs dose-dependently, albeit with lower sensitivity than heterodimeric scFv combinations (EC_{50} 4.3 μg ml^{-1} for G1–A7, 1.2 μg ml^{-1} for G1–B4; Fig. 3c). Tandem receptors comprising DARPin and scFv were as sensitive as their heterodimeric counterparts; for example, EC_{50} 0.31 μg ml^{-1} (versus 0.19 μg ml^{-1}) for the scFv–A7 receptor and 0.25 μg ml^{-1} (versus 0.12 μg ml^{-1}) for the scFv–B4 receptor. Induction with plasma led to increased signal intensities for all tandem receptors, even the previously non-responding G1–G1 (Fig. 3f). Thus, compact tandem receptors exhibit full functionality and mostly reflect the features of the corresponding heterodimeric receptors. The question of whether tandem configurations also allow for different ligands to be detected is addressed below.

Binding affinities of purified binders for xFDPs were measured by means of surface plasmon resonance (SPR) assuming a heterogenous binding model, which yielded two distinct binding constants; these were 169/244 nM for the scFv and 14/37 nM, 44/91 nM, 23/59 nM, and 602/1030 nM for DARPin receptors A6, A7, B4, and G1, respectively (Extended Data Fig. 4). We also confirmed that the binders are functional in a receptor context by employing...
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xFDPs, mirroring the higher affinity quantified by SPR measure - G1, and the scFv showed Rct values of 3.59 kΩ, 5.58 kΩ, 0.362 kΩ, and 0.62 kΩ, respectively. The heterodimeric B4 + scFv receptor showed strong activation (Rct 21.8 kΩ) by 1 μg ml⁻¹ xFDPS, mirroring the higher affinity quantified by SPR measurement (Extended Data Fig. 5 and Supplementary Fig. 8).

Determining fibrinogen activation as root cause of induction. To characterize the impact of potentially underrepresented xFDPS species in the initial DARPin selection as compared to the native plasma environment (Extended Data Fig. 1) and to exclude off-target receptor activation, we next investigated the receptor sensitivity to human plasma and tried to identify the active molecule(s) for AMBER B4, AMBER B4+scFv, AMBER G1-B4, and AMBER scFv-B4.

To this end, we characterized the receptor responses to purified fibrinogen (Fig. 4a), plasma fractions obtained by SEC and analyzed by mass spectrometry (Supplementary Fig. 9), and purified fragments D and E of fibrinogen (Supplementary Fig. 10). We also investigated the effect of plasmin on fibrinogen (Supplementary Fig. 11a). We found that activation of receptors by fibrinogen or plasma requires the presence of FCS containing active thrombin (Fig. 4b and Supplementary Fig. 12a) to produce active molecules composed of D–E or DD–E complexes (Extended Data Fig. 6). In line with the original publication, we found that the scFv moiety within AMBER G1-B4 and AMBER B4+scFv binds FDPs in a cross-linking-independent manner, and possible binding partners are fragment E as a single component or as a part of D–E or DD–E complexes (Extended Data Fig. 6).

We also investigated the effect of plasmin, its inhibitor tranexamic acid (TXA), and leech-derived peptide inhibitor of cross-linking factor XIIIa, tridegin, on coagulation-mediated receptor activation (Fig. 4c,d and Supplementary Fig. 13). In summary, we conclude that DARPin-B4 (present in AMBER B4 and AMBER B4+scFv) binds FDPs in a cross-linking-independent manner, and possible binding partners are fragment E as a single component or as a part of D–E or DD–E complexes (Extended Data Fig. 6). In line with the original publication, we found that the scFv moiety within AMBER B4+scFv binds cross-linked D-dimer protein in complex with fragment E (DD–E complex), which in turns binds to B4-DARPin (Extended Data Fig. 6). Receptors based on the scFv alone cannot detect D-dimers or coagulation (compare Fig. 3a,d). Nevertheless, we can build functional receptors that combine DARPin binders and the scFv.

The tandem receptor AMBER scFv-B4 detects larger partially digested and preferentially cross-linked FDPs while AMBER G1-B4 preferentially binds to non-cross-linked fragments (Fig. 4c,d and Extended Data Fig. 6). In conclusion, AMBERs in the analyzed set have different sensitivities and specificities towards fibrin degradation products, illustrating the versatility of the AMBER platform.
Even recently developed anti-coagulative drugs are constitutively active and hence increase the risk of bleeding, or can induce thrombocytopenia\textsuperscript{27,28}. An integrated cellular system able to measure efficacy and toxicity in parallel, using the DARPin and receptors developed here, could shorten the development time of new anti-coagulant therapies. We confirmed that our system responds to validated clinical samples and therapeutic outputs. AMBER\textsubscript{scFv-B4} can detect elevated levels of D-Dimers in patients' samples supplemented with heparin (P < 0.0001 versus negative control AMBER\textsubscript{scFv-B4} (Fig. 5a). Additionally, AMBER\textsubscript{scFv-B4} could detect both elevated D-dimer levels (Fig. 5b; P = 0.0049) and a hypercoagulable state\textsuperscript{30} leading to depletion of anti-coagulative factors\textsuperscript{31} in patients with disseminated intravascular coagulation (DIC) (Fig. 5b; P < 0.0001 versus normal D-dimer levels in samples without heparin).

**Generating designer cells to counteract coagulation.** To expand the functionality of the D-dimer-sensing receptors we constructed mammalian designer cells able to sense coagulation or elevated xFDPs and respond by secreting an anti-thrombotic protein, TNK (Extended Data Fig. 7a). The thrombin time of mHEK\textsubscript{scFv-B4} cells induced with xFDPs was assessed in a clinical laboratory and compared with that of the uninduced control (P = 0.0016). Values show mean ± s.d. of 10 (control) and 9 (elevated) patient samples (a), n = 4 patient samples for each condition (b), n = 4 independent samples (c). We performed statistical tests using a two-way analysis of variance with Šidák's multiple comparisons test (a,b) and a two-tailed, paired, parametric t-test (c).

**Detection of pathological events in patients' blood.** We next sought to validate our system using plasma from patients with elevated or physiological D-dimer concentrations. AMBER\textsubscript{scFv-B4} could detect pathological D-dimer levels in patients' samples supplemented with heparin (P < 0.0001 versus negative control AMBER\textsubscript{scFv-B4} (Fig. 5a). Additionally, AMBER\textsubscript{scFv-B4} could detect both elevated D-dimer levels (Fig. 5b; P = 0.0049) and a hypercoagulable state\textsuperscript{30} leading to depletion of anti-coagulative factors\textsuperscript{31} in patients with disseminated intravascular coagulation (DIC) (Fig. 5b; P < 0.0001 versus normal D-dimer levels in samples without heparin).
in the Golgi apparatus to deliver the two proteins to the supernatant in equimolar ratio so that reporter activity is correlated with potential therapeutic effect. Two stable monoclonal HEK-293T cell lines were selected and termed HEK<sub>AMBERA7</sub>/TNK (active receptor) and HEK<sub>AMBERB4</sub>/TNK (negative control) (Supplementary Fig. 14b). HEK<sub>AMBERB4</sub>/TNK cells showed a good correlation between TNK and Nluc in the supernatant of monoclonal HEK<sub>AMBERB4</sub>/TNK in response to xFDPs (Fig. 5c). The onset time was 6 h, within the expected limits of a transcription-based gene switch (Supplementary Fig. 14c,d). Notably, the system detected xFDPs at levels as low as 0.1 µg ml<sup>−1</sup>, well below the clinical disease threshold (0.5 µg ml<sup>−1</sup>), and was sensitive to plasma similar to transient transfections (Supplementary Fig. 14e,f).

**Preventing coagulation with hirudin-producing designer cells.** Hirudin<sup>12</sup> and heparin<sup>13</sup> or its derivatives are often used as anti-coagulants. Hence, we replaced the reporter construct used for inducible tenecteplase expression with an Nluc-p2a-hirudin sequence (secreted hirudin-HM2 from Pocillodella manillensis). We generated a stable monoclonal HEK-293T cell line (mHEK<sub>AMBERB4</sub>/Fv/hirudin) harboring the new reporter construct alongside AMBER<sub>B4</sub>/scFv. To assess the efficacy of the produced hirudin, we employed the AMBER<sub>B4</sub>-based coagulation-reporter system.

Monoclonal mHEK<sub>AMBERB4</sub>/Fv/hirudin cells were induced with xFDPs to co-express hirudin and Nluc enabling quantification of bioluminescence as a proxy for hirudin (Extended Data Fig. 9). After 24 h, the hirudin- and Nluc-containing supernatants were transferred to AMBER<sub>B4</sub>/Fv/SEAP cells. To profile the anti-coagulant effect of hirudin the reporter cell culture was supplemented with 0.5% (v/v) reconstituted human plasma. Purified hirudin and heparin were used as controls. Profiling hirudin production via bioluminescence and coagulation via AMBER<sub>B4</sub>-triggered SEAP expression enabled the quantification of the anti-coagulant impact of hirudin. Hirudin expression reduced clotting time by 10% (Fig. 5e), demonstrating functional secretion of the therapeutic protein.

**Detection of coagulation events in vivo.** In a proof-of-concept study, we assessed whether AMBER<sub>B4</sub>/Fv/scFv can be dose-dependently activated in mouse models of induced systemic coagulation and acute hepatic injury. Homodimeric AMBER<sub>B4</sub> and AMBER<sub>A7</sub> are activated by both human and mouse plasma in vitro, while AMBER<sub>B4</sub> is activated only by human plasma (Supplementary Fig. 15). Hence, in all mouse experiments we used the AMBER<sub>B4</sub>/Fv/scFv receptor alongside a STAT3-responsive Nluc reporter. The system was hydrodynamically...
injected via the tail vein to transduce the liver, and coagulation was induced 8 h later by injection of 500, 750, or 1000 U kg⁻¹ thrombin (Fig. 6a). After 24 h, expression of the reporter transgene correlated with D-dimer levels, indicating successful implementation of the AMBER(scFv) system in vivo (Fig. 6b). Next, we evaluated the system in a mouse model of acute hepatic injury⁴³ caused by acetaminophen (N-acetyl-p-aminophenol (APAP)), a leading cause of acute liver damage in humans⁴⁵. APAP-induced liver injury leads to activation of coagulation and hepatic fibrin deposition within 2–6 h in mice. Measurements of blood levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) at 8 h and 12 h after intraperitoneal APAP injection confirmed liver damage (Fig. 6c), and elevated levels of NLuc demonstrated that the AMBER sensor was activated (Fig. 6d).

### Discussion

In this work we have combined DARPin selection with our GEMS technology, and we show that the resulting AMBER platform enables the generation of programmable receptors with customizable sensitivity and specificity by screening a vast space of possible epitopes. Such versatility is unprecedented in a receptor system for soluble ligands. We confirmed that this modular receptor system works as expected for FDPs, which we chose as markers with high clinical relevance for proof of concept. Coagulation was detected at an early stage before the build-up of insoluble fibrin by employing the AMBER(scFv) system, and we could detect early as well as late biomarkers of thrombotic events by using an integrated sensor incorporating a previously characterized scFv (AMBER(scFv-B4), or AMBER(scFv-B4)). Furthermore, AMBER receptors were coupled to therapeutic protein output, either tenecaplose to boost clot lysis or hirudin to prevent further coagulation, in designer cell lines. Secretion of a therapeutic protein alongside a bioluminescent reporter provided a direct correlation of reporter activity with expression of the therapeutic protein. Finally, we established that the system is applicable for the detection of thrombotic events in patients, as well as in mouse models in which coagulation is induced either systemically, mimicking DIC, or triggered locally by APAP-induced liver injury. These results suggest that the AMBER pipeline has translational potential for cell-therapy approaches to treat recurrent thrombosis or embolism.

In addition, the selected anti-FDP DARPins may be useful to expand new diagnostic tools or therapeutic agents, by exploring the available epitope landscape for the development of ELISA-like assays or DARPin-coupled fibrin-targeted drugs for clot lysis. Owing to its shorter half-life, fragment E (as presumably targeted by some of the DARPins in this study) might provide a more precise insight into the progression of thrombotic diseases as compared to other degradation products such as D-dimers⁴⁶,⁴⁷.

Notably, not all hits from the initial DARPin selection turned out to be good candidates for building a functional receptor, highlighting the importance of a diverse library as input for the AMBER pipeline—especially since sandwich pairs, both having sufficient affinity, and a suitable relative disposition of epitopes are required. Indeed, for induction by plasma, about 16% of combinations with a known scFv and 40% of DARPin-only receptors were active in a heterodimeric receptor configuration. Since about 83% of these DARPin-only receptors are based on one of four of the initially characterized 19 DARPins, the overall success rate in finding suitable binders was 21%. With a symmetric target, the generation of homodimeric receptors is much more likely, and we thus expect the likelihood of finding such receptors for monomeric targets to be lower. Nevertheless, the GEMS platform has been successfully used in combination with pre-existing binders to detect the monomeric 26kDa cancer antigen PSA with the use of two scFvs⁴⁸.

Confirmation of the binding of xFDPs to homodimeric receptors by EIS means that the OFF-type switching observed in the reporter gene assays (Fig. 3a) can indeed be attributed to inactivation of homodimeric receptors by ligand binding. Also, binding of xFDPs is much stronger in a heterodimeric setting (B4 + scFv) than in the homodimeric setup (B4 or scFv alone), indicating a non-linear effect of receptor–ligand interaction, possibly owing to increased avidity as suggested by SPR quantifications (Extended Data Figs 8 and 9).

As elaborated on earlier, we expect the number of working combinations of FDP binders that satisfy the design constraints of the receptor architecture to be low owing to size restrictions. From a reported crystal structure of activated EpoR (PDB ID: 1EER), as well as the results of the GFP-AMBER experiments we can estimate the distance between two epitopes to range from ~8 nm (distance between the ends of the D1 domain of activated EpoR comprising the linker length and the size of GFP) to ~3 nm (two times half the length of the GFP barrel (PDB ID: 5B61) in the GFP–GFP fusion without any linker). Of course, the geometry of the receptor–ligand interaction should be taken into account⁴⁹, but different binder types would allow for different geometries.

While one can build receptors using pre-existing binders, an integrated pipeline to check a receptor library for activity in cell culture greatly extends the options, is easy to parallelize and is time- and cost-efficient. Therefore, we expect the versatile and highly streamlined DARPin technology in combination with the AMBER technology to provide access to large numbers of receptors for many targets, including soluble cancer antigens, cytokines or even cell-surface epitopes. AMBER might be especially useful for targets for which no natural receptor is known and for which available sequences of scFvs or other binders are limited, especially since sandwich pairs are modular. Switching of the signaling domains of the underlying receptor scaffold has already been reported and could be used to accelerate research in systems and stem cell biology to rewire endogenous signaling cascades.

The ribosome display technology coupled with the DARPin libraries used in this work provides a powerful method to generate highly diverse sets of binders through enrichment of high-affinity binders in a cell-free setup, and expanding the receptor platform to include other types of binders with different epitope preferences further broadens the range of possible epitopes.

While the DARPins generated in our screen have a concave interaction surface, other scaffolds mainly interact via protruding loops. Hence, we believe that different binder types can complement each other in sandwich pairs to facilitate the generation of AMBERs. The benefits of combining different binder types are already apparent in the present study in terms of the clear change in specificity observed upon employing an scFv in combination with a DARPin.

### Online content

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### References

1. Scheller, L. & Fussenegger, M. From synthetic biology to human therapy: engineered mammalian cells. *Curr. Opin. Biotechnol.* 58, 108–116 (2019).

2. Mohammed, S. et al. Improving chimeric antigen receptor-modified T cell function by reversing the immunosuppressive tumor microenvironment of pancreatic cancer. *Mol. Ther.* 25, 249–258 (2017).

3. Schwarz, K. A., Daringer, N. M., Dolberg, T. R. & Leonard, J. N. Rewiring human cellular input–output using modular extracellular sensors. *Nat. Chem. Biol.* 13, 202–209 (2017).
23. Grieger, J. C. & Samulski, R. J. Packaging capacity of adeno-associated virus

22. Brown, J. H., Volkmann, N., Jun, G., Henschen-Edman, A. H. & Cohen, C.

21. Kollman, J. M., Pandi, L., Sawaya, M. R., Riley, M. & Doolittle, R. F. Crystal

20. Rothbauer, U. et al. Targeting and tracing antigens in live cells with

19. Hansen, S. et al. Design and applications of a clamp for green fluorescent

18. Schilling, I., Schoppke, J. & Pluckthun, A. From DARPinS to LoopDARPinS: novel LoopDARPin design allows the selection of low picomolar binders in a single round of ribosome display. *J. Mol. Biol.* 426, 691–721 (2014).

17. Uchanski, T. et al. An improved yeast surface display platform for the screening of nanobody immune libraries. *Sci. Rep.* 9, 382 (2019).

16. Pluckthun, A. Designed ankyrin repeat proteins (DARPins): binding proteins for research, diagnostics, and therapy. *Annu. Rev. Pharmacol. Toxicol.* 55, 489–511 (2015).

15. Dreier, B. & Pluckthun, A. Rapid selection of high-affinity binders using ribosome display. *Methods Mol. Biol.* 805, 3–28 (2012).

14. Dreier, B. & Pluckthun, A. Rapid selection of high-affinity binders using ribosome display. *Methods Mol. Biol.* 805, 261–286 (2012).

13. Pluckthun, A. Designed ankyrin repeat proteins (DARPins): binding proteins for research, diagnostics, and therapy. *Annu. Rev. Pharmacol. Toxicol.* 55, 489–511 (2015).

12. MacCallum, R. M., Martin, A. C. & Thornton, J. M. Antibody–antigen interactions: contact analysis and binding site topography. *J. Mol. Biol.* 262, 732–745 (1996).

11. Adam, S. S., Key, N. S. & Greenberg, C. S. D-dimer antigen: current concepts and future prospects. *Blood* 113, 2878–2887 (2009).

10. Weng, J., Xu, X. & Doolittle, R. F. Crystal structure of human fibrinogen. *Biochemistry* 48, 3877–3886 (2009).

9. Brown, J. H., Volkman, N., Jun, G., Henschen-Edman, A. H. & Cohen, C. The crystal structure of modified bovine fibrinogen. *Proc. Natl Acad. Sci. USA* 97, 85–90 (2000).

8. Grieger, J. C. & Samulski, R. J. Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. *J. Virol.* 79, 9933–9944 (2005).

7. Laroche, Y. et al. Characterization of a recombinant single-chain molecule comprising the variable domains of a monoclonal-antibody specific for human fibrin fragment D-dimer. *J. Biol. Chem.* 266, 16343–16349 (1991).
**Methods**

Chemicals and proteins. Native human D-dimer protein (ab98311), recombinant hirudin protein (ab201396) and native human plasmin (ab90928) were purchased from Abcam. *Trans*-4-((Aminomethyl)cyclohexanecarboxylic acid (transamidic acid, TXA, 85763), heparin sodium salt from porcine intestine (H3149), lophophorized human plasmin (P9523), active thrombin from human plasma (SRP6557), and fibrin from human plasma (F5386) were purchased from Sigma-Aldrich. Human fibrinogen fragment E (HCI-0159E) and human fibrinogen fragment D (HCI-0150D) were purchased from Haematologics Technologies. Soluble human fibrin (FIB-S-1.0MG) was purchased from Molecular Innovations Trigègn (op0062) was purchased from Covaleda S.A.

Selection and screening of DARPin binders specific for FDP. To generate DARPin binders for human FDP protein, FDPs were biotinylated using EZ-Link NHS-Biotin (ThermoFisher Scientific) and the biotinylated target protein was immobilized on streptavidin-coated beads. Flag-tagged DARPin fragments were expressed from plasmids DARPin for Sera-Mag neutravidin-coated beads (GE), depending on the selection round. Ribosome display selections were performed essentially as described, using a semi-automatic KingFisher Flex MTP96 well platform.

For the present work, we used an established, optimized, fully synthetic DARPin library in which the backbone of the DARPin scaffold remained constant and the amino acids forming the target-interacting surface were randomized. The design strategy and characteristics of this library, which has a theoretical diversity of 3.8 × 10^23 unique sequences, have been reviewed elsewhere. Because of the high diversity of the library and the limited number of actual molecules present in each ribosome display run (estimated to be 'only' 10^9), essentially no duplicates are screened, as well as it also allows for repeated selections against the same target to yield different binders.

We started screening with a synthetic library comprised of DARPins with three internal repeat domains and distinct N-terminal and C-terminal capping repeats (N3C-DARPins), where the terminal repeats were designed with or without randomized or predicted binding interfacial residues. A full-length mRNA was synthesized by RT-PCR using a human FDP library. The selected mRNA is used in a subsequent cycle to further increase the affinities of DARPins in the pool. This sequence of reactions allows for the selection of the highest affinity binders from the initial library, as described below.

The fully synthetic library consists of N3C-DARPins with three randomized internal repeats, containing a mixture of non-randomized and randomized N-terminal and C-terminal capping repeats. Selections were performed over four rounds with decreasing concentrations of competitor for the first three cycles, an off-rate selection using non-biotinylated xFDPs in the third cycle and a recovery phase with less stringent conditions in the fourth cycle.

The final enriched pool was cloned as fusions into a bacterial pQ3E0 derivative vector with a N-terminal MRGSH and C-terminal FLAG tag via unique BamHI and HindIII sites, containing lacIq for expression control. After transformation of each plasmid, cell-free extract was generated. Since the mRNA lacks a stop codon, translation is stalled at the ribosome. Consequently, the mRNA is attached through the ribosome and capped from the tRNA. Because of extra residues at the C terminus (the ‘tail’) this polypeptide folds into a functional DARPin that can bind to the corresponding nascent peptide. Because of extra residues at the C terminus (the ‘tail’) this polypeptide folds into a functional DARPin that can bind to the corresponding nascent peptide.

For stable cell line generation, HEK-293T cells were used. The selection of recombinant DARPin binders was performed using the Sleeping Beauty transposon system. Donor plasmids bearing a selection marker (resistance genes for puromycin (pu), blasticidin (br) or zeocin (ze)) driven by the constitutive promoter and flanked by recognition sites of the Sleeping Beauty transposase were co-transfected with an expression plasmid for Sleeping Beauty transposase. For selection of stable clones, the cell culture medium was supplemented with puromycin (2 µg ml^-1), blasticidin (4 µg ml^-1) or zeocin (20 µg ml^-1) or combinations of them, depending on the type of plasmids to be integrated.

**Plasmid preparation.** A comprehensive list of all plasmids used in this study is provided in Supplementary Table 1.

Plasmids were generated using conventional molecular cloning techniques. Polymerase chain reaction (PCR) was performed using Phusion polymerase (F530, ThermoFisher Scientific) or Q5 high-fidelity polymerase (M0491, New England Biolabs, NEB) following the manufacturers’ recommendations. Cleavage of PCR products and plasmids was done with restriction endonucleases (New England Biolabs NEB, HF enzymes were used if applicable) and ligated with T4 DNA ligase (E1001, ThermoFisher Scientific).

**SDS-PAGE and Coomassie staining.** Samples were heated for 5 min at 70°C before loading. Up to 20 µl sample was loaded per well of a 12-well or 15-well polyacrylamide gel (Bolt 4–12% Bis-Tris Plus gels, Invitrogen). SDS-PAGE was performed by using 1X Bc MOPS SDS Running Buffer (Invitrogen) in a XCell SureLock Mini-Cell (Invitrogen) at 200 V. Following the run, the gel was stained with Coomassie Brilliant Blue G250 for >1 h at room temperature. Gels were washed and destained in water for several days before mass-spectrometric analysis.

**Cell culture.** HEK-293T/17 (HEK-293T) cells (ATCC CRL-11268) were purchased from the American Type Culture Collection and grown in high-glucose Dulbecco’s modified Eagle’s medium containing Glutamax (DMEM, high glucose, GlutaMAX Supp, 61965026, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS, F5274, Sigma-Aldrich) at 37°C in a humidified incubator with 7.5% CO₂ atmosphere. Cells were grown in 10 ml DMEM at a seeding density of 1.5 × 10⁴ cells in a 10-cm culture dish and kept at <80% confluency.

For transfection, cells were seeded in 15 ml DMEM supplemented with 1X penicillin/streptomycin (L0022, Biowest) in 96-well culture plates (15,000 cells per well). Stable HEK-293T cells were generated using the Sleeping Beauty transposon system. Donor plasmids bearing a selection marker (resistance genes for puromycin (pu), blasticidin (br) or zeocin (ze)) driven by the constitutive promoter and flanked by recognition sites of the Sleeping Beauty transposase were co-transfected with an expression plasmid for Sleeping Beauty transposase.

For selection of stable clones, the cell culture medium was supplemented with puromycin (2 µg ml^-1), blasticidin (4 µg ml^-1) or zeocin (20 µg ml^-1) or combinations of them, depending on the type of plasmids to be integrated.

**Transfections and stable cell line generation.** HEK-293T cells were seeded from the same batch on the evening before transfection in the same plate format. For most reporter assays, transfection was done in a 96-well plate by mixing 200 ng plasmid DNA and 1.2 µl of 1 mg ml^-1 polyethyleneimine (PEI, 24765-1, Polysciences) per well in FCS-free DMEM if not indicated otherwise. The mixture was incubated for 5–10 min, added to each well and incubated with the cells overnight. For all transient expression experiments, 2 ng each receptor plasmid was mixed with 30 ng plasmid pLS and 300 ng each integration vector were mixed with 200 ng Sleeping Beauty expression vector pTS395 and 200 ng pDF101. A comprehensive list of plasmids used in each experiment is provided in Supplementary Table 2.

For stable cell line generation, 6-well plates were used. Here 250,000 cells per well were seeded and transfected 24 h later. A total of 1 µg plasmid DNA was mixed with 1.2 µl 200 ng plasmid pLS. Stable HEK-293T cells were generated using the Sleeping Beauty transposon system. Donor plasmids bearing a selection marker (resistance genes for puromycin (pu), blasticidin (br) or zeocin (ze)) driven by the constitutive promoter and flanked by recognition sites of the Sleeping Beauty transposase were co-transfected with an expression plasmid for Sleeping Beauty transposase.

For selection of stable clones, the cell culture medium was supplemented with puromycin (2 µg ml^-1), blasticidin (4 µg ml^-1) or zeocin (20 µg ml^-1) or combinations of them, depending on the type of plasmids to be integrated.

**Reporter assays.** To measure the activity of SEAP, the increase of absorbance at 405 nm owing to hydrolysis of para-nitrophenyl phosphate (pNPP) in the cell culture supernatant was followed (1 reading per minute for 25 min). To this end, 60–100 µl supernatant was collected from each well and heat-inactivated for 30 min at 65°C. One hundred eighty microilters of assay reagent containing 100 µl 2X SEAP buffer (20 mM homogargin, 1 mM MgCl₂, 2% (v/v) diethanolamine, pH 9.8) and 0.2 µM pNPP substrate solution (100 µM, in 2X SEAP buffer) and 5 µl water were added to 20 µl heat-inactivated supernatant. The absorbance at 405 nm at 37°C was monitored with a Tecan M1000 multipurpose reader (Tecan AG) and used to calculate SEAP activity.

Cell culture supernatant was also used to determine the activity of secreted nano-hydroxynitrilase with the Nano-Glo Luciferase Assay System (V1110, Promega). To this end, 7.5 µl cell culture supernatant was mixed with 7.5 µl buffer/substrate mix per well in a 384-well black-well plate. Luminescence of the reporter was measured using a Tecan M1000 multipurpose reader (Tecan AG).

**Sequence analysis.** Phylogenetic analysis of amino acid sequences was done with a simple phylogeny tool. Sequence alignments were performed using Clustal O (https://www.ebi.ac.uk/Tools/msa/clustalo/). Pairwise identity calculation was carried out using the EMBL-EBI Clustalo server employing the Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). All algorithms are part of the EMBL-EBI search and sequence analysis tools API.
GFP fusion protein production and purification. BL21-Gold (DE3) cells were transfected with plasmids containing the expression units controlled by a lac operator. After induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside, expressed cells were collected for 16 h at 18 °C and harvested by centrifugation at 4,000 rpm at 4 °C. Cell pellets were then resuspended in 50 mM Tris-HCl (pH 8.0), 400 mM NaCl (TBS400), supplemented with 3 mg/ml lysozyme (Sigma-Aldrich) and 100 µg/ml DNase I (Roche), and lysed by sonication. The lysates were centrifuged (21,000 rpm, 30 min, 4 °C) and the supernatants were applied to Ni-NTA Superflow (Qiagen) metal ion affinity columns (4 ml). Each column was washed with 15 column volumes of 50 mM Tris-HCl (pH 8.0), 20 mM imidazole supplemented with 400 mM NaCl, 1 M NaCl or 20 mM NaCl. The proteins were eluted in 5 column volumes PBS (pH 7.4), 500 mM imidazole. The eluted proteins were transferred into dialysis tubes with a molecular weight cutoff of 6,000–8,000 Da, supplemented with 100 µg/ml TEV protease (GenScript), and dialyzed for 36 h at 4 °C in phosphate-buffered saline, TEV protease–cleaved His-tags and non-cleared proteins were removed by applying the dialysed samples to Ni-NTA Superflow metal ion affinity columns (4 ml). The flow-through fraction was collected and concentrated by ultrafiltration (Amicon Centrifugal Filter Units, Millipore). The concentrated protein was then run through a Superdex 75 10/300 GL column for homogeneity and purity. Protein concentrations were determined by UV-Vis spectroscopy and purity was confirmed by SDS-PAGE analysis.

Size-exclusion chromatography of reconstituted plasma. Reconstituted human plasma was filtered through a 0.2-µm syringe filter to clear liposomes and loaded on a self-packed Superdex 30 column equilibrated with D-PBS containing 2 mM EDTA to prevent clotting in the column. The run was performed in the same buffer and a total of 133 fractions of 1 ml were collected. Selected fractions were further tested for their activity in cell culture as well as analyzed with SDS-PAGE and western blotting.

Mass spectrometry. In-gel digestion of relevant SDS-PAGE bands was performed by using trypsin in 100 mM ammonium bicarbonate buffer. Samples were purified by using C18 columns, dried, and resuspended in 20 µl of a suitable buffer. Concentrations were adjusted to be approximately 0.1 µg/ml and 1 µg of the solution was subjected to mass-spectrometric analysis on an Orbitrap Elite system.

Surface plasmon resonance measurement. Affinities of soluble binders were assessed using SPR with a CMD200M chip on a Biacore T200 (GE Healthcare). Affinities of soluble binders were assessed using SPR using a CMD200M chip on a Biacore T200 (GE Healthcare). Surface plasmon resonance measurement. Affinities of soluble binders were assessed using SPR with a CMD200M chip on a Biacore T200 (GE Healthcare). Surface plasmon resonance measurement. Affinities of soluble binders were assessed using SPR with a CMD200M chip on a Biacore T200 (GE Healthcare). Surface plasmon resonance measurement. Affinities of soluble binders were assessed using SPR with a CMD200M chip on a Biacore T200 (GE Healthcare). Surface plasmon resonance measurement. Affinities of soluble binders were assessed using SPR with a CMD200M chip on a Biacore T200 (GE Healthcare). Surface plasmon resonance measurement. Affinities of soluble binders were assessed using SPR with a CMD200M chip on a Biacore T200 (GE Healthcare).

Electrochemical impedance spectroscopy-based sensor assay. HEK-293T cells were transfected with the plasmids described above, either in homodimeric or heterodimeric receptor chains. System-printed Au electrodes (DS 220 AT, Metrohm) organized in the form of a three-electrode system were deployed to carry out the sensing measurements using a CH Instruments Electro-chemical Workstation (USA). The surface of the working electrode was modified with 0.01 µg/ml multiwall carbon nanotubes (Sigma) and polyethylene glycol (Sigma) (200,000 molecular weight) in order to facilitate cell seeding and limit cell migration to the counter or reference electrodes. Transfected cells were seeded at a concentration of 8,000 cells per milliliter in standard culture medium. Cells were incubated on the electrodes at 37 °C overnight to ensure proper adherence. During the experiment, the cell-covered electrodes were exposed to xPDAs at concentrations of 1.25, or 10 µM. Measurements were carried out in the presence of 5 mM solution of the redox couple Fe(CN)63−/4− (Sigma). The measurements were performed in EIS mode, wherein a potential of +0.2 V was maintained between the working and reference electrodes (Ag/AgCl). The acquired Nyquist plots for homodimeric and heterodimeric interactions were fitted using two different modified Randle’s models for the indicated concentrations. The expression of NLuc reporter in the supernatant was measured after 8 h.

Mouse experiments. Wild-type male 6-week-old BALB/c mice obtained from East China Normal University (ECNU) Laboratory Animal Center were used and kept in a pathogen-free environment. Mice were kept in a 12 h light/12 h dark cycle at room temperatures ranging between 18 °C and 23 °C and humidities between 40% and 60%.

Thrombin-mediated transgene expression in mice. Two milliliters (10% of the body weight in grams) Ringer’s solution (147 mM NaCl, 4 mM KCl, 1.13 mM CaCl2), containing approximately 360 µg plasmids encoding the thrombin sensor (pT8S64 (PhCMV-DARPinB4-EpoR-pA), 30 µg pTS922 (PhCMV-scFv-EpoR pA)), 30 µg pH6 (PhCMV-NLuc-pA-PhCMV-STAT3-3A), 300 µg was hydrodynamically injected into each mouse via the tail vein within 3–5 s. After injection, mice were randomly divided into four groups. Eight hours after plasmid injection, mice were injected with thrombin from bovine plasma (Beyotime, cat. no. 2594047) at doses ranging from 0 to 1,000 U kg−1 via the tail vein to induce thrombin formation. At 16 h after thrombin injection, plasma samples were obtained by centrifugation (2,700g for 10 min) of clotted blood in blood collection tubes (BD, cat. no. 365974) for analytical assays. Dimeric levels were measured using a Mouse D-Dimer ELISA Kit (Elabscience, cat. no. E-EL-M4004) following the manufacturer’s instructions, and plasma Nanoluc levels were measured using a Nano-Glo Luciferase Assay Kit (Promega, cat. no. N1120) according to the manufacturer’s instructions.

AMBER activation in an APAP-induced acute hepatic injury mouse model. Two milliliters (10% of the body weight in grams) Ringer’s solution (147 mM NaCl, 4 mM KCl, 1.13 mM CaCl2), containing approximately 360 µg plasmids encoding the thrombin sensor (pT8S64 (PhCMV-DARPinB4-EpoR-pA), 30 µg pTS922 (PhCMV-scFv-EpoR-pA)) and 300 µg pH6 (PhCMV-NLuc-pA-PhCMV-STAT3-3A) was hydrodynamically injected into mice via a tail vein within 3–5 s. After the injection, mice were immediately fasted and randomly divided into two groups. Fifteen hours after plasmid injection, fasted mice were intraperitoneally injected with APAP (0 or 160 mg kg−1, Absin, cat. no. abs815915–500mg). Blood AST, ALT and Nluc levels were measured at 8 h and 12 h after APAP injection, and 12 and 24 h hours after APAP injection, blood samples were taken, allowed to clot, and centrifuged (2,700g for 10 min) in blood collection tubes (BD, cat. no. 365974) for analytical assays. Plasma AST and ALT levels were measured using an AST detection kit (KHB; SHFDA: 20114200181) and an ALT detection kit (KHB; SHFDA: 20172406016) according to the manufacturer’s instruction. Plasma Nluc levels were measured using a Nano-Glo Luciferase Assay Kit (Promega, cat. no. N1120) according to the manufacturer’s instructions.

Statistics. Calculations were performed in Microsoft Excel for Mac 16.50. All statistical analyses were done with GraphPad Prism 9.1. Representative graphs showing n=3 biologically independent samples are presented as bar diagrams + s.d. Unless indicated otherwise, no statistical analysis was performed. Plots in Fig. 3 show the mean ± s.e.m. of n= 3 independent experiments performed in triplicate. To determine the level of significance in Fig. 5a,b, a two-way analysis of variance was performed with Sidák’s multiple comparisons test. For the animal experiments in Fig. 6b, each treatment group consisted of randomly selected 6 to 8 male mice. Sample results are expressed as mean ± s.e.m. The statistical parameters were calculated to be as follows. For Fig. 5a, AMBER3−3A, adjusted P < 0.0001, t = 6.396, degrees of freedom = 34.00; AMBER3−3A, adjusted P not significant, t = 0.7959, degrees of freedom = 34.00. For Fig. 5b, left, 0%, adjusted P > 0.0009, t = 0, degrees of freedom = 12; 5%, adjusted P = 0.0049, t = 3.821, degrees of freedom = 12. For Fig. 5b, right, 0%, adjusted P > 0.0009, t = 0.12, degrees of freedom = 12; 1%, adjusted P < 0.0001, t = 0, degrees of freedom = 12. To determine the level of significance in Fig. 5e and Fig. 6b, two-tailed unequal parametric t-tests were performed for the animal experiments.
Fig. 6b, d, each treatment group consisted of randomly selected mice (n = 5–6). The results are expressed as mean ± s.e.m. The parameters for t-tests were calculated to be for Fig. 6i: P = 0.0016, t = 7.585, degrees of freedom = 4. For Fig. 6b (NLuc reporter), 500 U kg⁻¹ versus 0 U kg⁻¹, P < 0.0001, t = 6.597, degrees of freedom = 9; 750 U kg⁻¹ versus 0 U kg⁻¹, P = 0.0004, t = 5.194, degrees of freedom = 10; 1,000 U kg⁻¹ versus 0 U kg⁻¹, P < 0.0001, t = 6.224, degrees of freedom = 10. For Fig. 6b (D-dimer levels), 500 U kg⁻¹ versus 0 U kg⁻¹, P < 0.0001, t = 10.85, degrees of freedom = 9; 750 U kg⁻¹ versus 0 U kg⁻¹, P = 0.0085, t = 5.055, degrees of freedom = 10; 1,000 U kg⁻¹ versus 0 U kg⁻¹, P = 0.0002, t = 5.625, degrees of freedom = 10. For Fig. 6d for ALT, 8 h, adjusted P = 0.002039, t = 5.936, degrees of freedom = 6; 12 h, adjusted P = 0.002999, t = 4.8, degrees of freedom = 6; for AST, 8 h, adjusted P = 0.000664, t = 8.363, degrees of freedom = 8; 12 h, adjusted P = 0.000736, t = 5.699, degrees of freedom = 7; for NLuc reporter, 8 h, adjusted P = 0.001878, t = 6.03, degrees of freedom = 6; 12 h, adjusted P = 0.003533, t = 4.642, degrees of freedom = 6.

Ethics. Animal experiments were approved by East China Normal University (ECNU) Animal Care and Use Committee and were conducted in accordance with the Ministry of Science and Technology of the People’s Republic of China guidelines. The protocol (ID: m20191212) was approved by ECNU Animal Care and Use Committee. All mice were killed after completion of the experiments. Experiments involving patients’ samples were approved by the Medical Ethics Committee of Yangpu Hospital affiliated with Tongji University (request ID: LL-2018-SCI-003) and the Ethikkommission Nordwest und Zentralschweiz (EKNZ) (request ID: 2021-01998). All human participants agreed to the use of their blood samples for research purposes and expressed their consent in writing.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

DNA sequences are publicly available at benchling.com (https://benchling.com/tobstr/2/zjVVweqtstrittmatter-et-al-2022-natchembio-amber) and at Genbank (ON681641–ON681702). Genbank accession numbers are provided with the plasmid table (ON681641–ON681702; Supplementary Table 1). Raw data for each figure is provided as .xlsx files alongside the manuscript. Source data are provided with this paper.

References

38. Schilling, I., Schoppel, J., Sauer, E. & Pluckthun, A. Co-crystallization with conformation-specific designed ankyrin repeat proteins explains the conformational flexibility of BCL-W. J. Mol. Biol. 426, 2346–2362 (2014).
39. Zahnd, C., Sarkar, C. A. & Pluckthun, A. Computational analysis of off-rate selection experiments to optimize affinity maturation by directed evolution. Proteins Eng. Des. Sel. 23, 175–184 (2010).
40. Madeira, E. et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 47, W636–W641 (2019).
41. Kowarz, E., Loscher, D. & Marschalek, R. Optimized Sleeping Beauty transposons rapidly generate stable transgenic cell lines. Biotechnol. J. 10, 647–653 (2015).

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Author contributions

T.S., L.S. and M.F designed the project, T.S., Y.W., A.B., P.C.F., P.G.R., analyzed data and wrote the manuscript; J.V.S., D.T., A.P., H.Y., and M.F. supervised the project and wrote the manuscript.

Competing interests

T.S., L.S. and M.F are inventors on a patent describing the GEMS platform (US Patent App. 16/737,076). A.P. holds patents on DARPin technology (US8513164B2, US9365629B2, US7417130B2).

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41589-022-01095-3.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41589-022-01095-3.
Correspondence and requests for materials should be addressed to Martin Fussenegger.

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Extended Data Fig. 1 | Overview of processes involved in fibrinolysis. Overview of processes involved in fibrinolysis. Fibrinogen forms a head-to-head dimer via the E-domain (blue) that is covalently linked via disulfide bonds. Upon activation with thrombin, fibrinogen oligomerizes via interactions of its D-domains (green) with the E-domain to form an insoluble non-cross-linked fibrin mesh. The mesh is stabilized by factor XIIIa, which introduces isopeptide bonds between adjacent D-domains. Fibrinolysis is mediated by plasmin, which cleaves fibrin into smaller soluble fragments. Depending on whether the fibrin was cross-linked prior to plasmin digestion, different types of fragments arise. Degradation products include fragment E comprising the E-domain of fibrinogen (blue), fragment D (green) and cross-linked D-dimers (green, indicated by orange crosses), as well as complexes thereof. Many of these products contain the same epitopes.
Extended Data Fig. 2 | Ribosome-display-based DARPin selection. Overview of ribosome-display-based DARPin selection to generate novel binding domains. An optimized DARPin library containing a mixture of N3C-DARPin with randomized and non-randomized N- and C-caps is transcribed to mRNA, which is translated in vitro. Since the mRNA lacks a stop codon, the ribosome is stalled on the mRNA with the nascent peptide still attached via a fused tether upon completion of translation. The fully folded DARPin can now bind to FDPs coupled to streptavidin-coated beads via their biotinylated lysine residues. FDP-DARPin-ribosome-mRNA complexes are washed and subsequently dissolved. The resulting mRNA is reverse-transcribed to yield an enriched plasmid library, which is subjected to three additional rounds of the same selection cycle. The products of the final round are transformed into \textit{E. coli} for single clone screening.
Extended Data Fig. 3 | Screening of homodimeric receptors in HEK-293T. Screening of homodimeric receptors in HEK-293T. Cells were incubated with 0.5 µg/mL xFDPs or 0.5 % (v/v) reconstituted human plasma in complete DMEM containing 10 % FCS for 24 h, and SEAP reporter activity was measured. RR120 was included as a negative control and constitutive expression of SEAP was used as a reference. All values are means ± SD of n = 3 independent samples.
Extended Data Fig. 4 | SPR measurements of scFv and DARPin binders. Affinities of binders towards the xFDP preparation used for DARPin selection were assessed by surface plasmon resonance (SPR) measurements on a Biacore T200 (GE Healthcare) using a CMD200M chip coated with 205 RU of xFDP protein. Due to the symmetric structure of the target molecules and thus a potential interaction between the binders on the ligand, we stipulated a heterogenous binding modality, resulting in two distinct binding constants. Affinities of G1-DARPin were up to 10- to 50-fold lower than those of other DARPins may explain the poor performance of G1-DARPin in a receptor context. Duplicates of full kinetic measurements were performed and fitted with a heterogenous ligand (hetlig) model using the BIAevaluation software (GE Healthcare). Flow cell 1 (FC1) served as a reference for FC2 and was coated with 205 RU of xFDP via NHS linkers. Each concentration was measured twice in ascending and descending order.
Verification of binding of xFDPs to receptors by electrochemical impedance spectroscopy (EIS). Binding of xFDPs to receptors was verified by electrochemical impedance spectroscopy (EIS). A three-electrode system was employed (Metrohm DS - 220AT) to sense changes in the charge transfer resistance ($R_{ct}$) of the redox couple $[\text{Fe(CN)6}]^{3-}/[\text{Fe(CN)6}]^{4-}$ caused by binding of xFDPs to the receptors which insulates the electrode and hampers electron flow. Higher $R_{ct}$ values indicate an increase in resistance and are indicative of higher affinity of the receptor, which translates to increased sensitivity.

a) Cells transfected with receptors were incubated in the presence of $[\text{Fe(CN)6}]^{3-}/[\text{Fe(CN)6}]^{4-}$, and changes in $R_{ct}$ were recorded upon addition of 1 µg/ml of xFDPs to

b) homodimeric receptors

c) heterodimeric receptors

d) $R_{ct}$ of homodimeric receptors

e) $R_{ct}$ of heterodimeric receptor

Extended Data Fig. 5 | Verification of binding of xFDPs to receptors by electrochemical impedance spectroscopy (EIS). Binding of xFDPs to receptors was verified by electrochemical impedance spectroscopy (EIS). A three-electrode system was employed (Metrohm DS - 220AT) to sense changes in the charge transfer resistance ($R_{ct}$) of the redox couple $[\text{Fe(CN)6}]^{3-}/[\text{Fe(CN)6}]^{4-}$ caused by binding of xFDPs to the receptors which insulates the electrode and hampers electron flow. Higher $R_{ct}$ values indicate an increase in resistance and are indicative of higher affinity of the receptor, which translates to increased sensitivity. a) Cells transfected with receptors were incubated in the presence of $[\text{Fe(CN)6}]^{3-}/[\text{Fe(CN)6}]^{4-}$, and changes in $R_{ct}$ were recorded upon addition of 1 µg/ml of xFDPs to b) homodimeric or c) heterodimeric receptors. b) Homodimeric receptors based on DARPin A6, A7, B4, G1 and the scFv showed $R_{ct}$ values of 3.59 kΩ, 1.48 kΩ, 5.58 kΩ, 0.362 kΩ, and 0.62 kΩ, respectively. c) The heterodimeric B4 + scFv receptor showed a strong activation ($R_{ct} = 21.88$ kΩ). $R_{ct}$ values for 1, 2.5 and 10 µg/ml of xFDPs measured for d) homodimeric receptors and e) B4 + scFv heterodimeric receptor seem to saturate at 2.5 µg/ml for all receptors, except for the homodimeric DARPin A7 receptor. Marginal increase in $R_{ct}$ of RR120 control receptor can be attributed to non-specific binding. All values are means ± SD of $n = 3$ independent samples.
**Extended Data Fig. 6 | Summary of suggested receptor specificities.** Summary of suggested receptor specificities. AMBER$_{B4}$ may preferentially bind to fragment E of fibrin, AMBER$_{B4/scFv}$ may bind to cross-linked D-dimers complexed with fragment E (DD-E complex) or fragment E alone (because some parts are also presented in the AMBER$_{B4}$ configuration), AMBER$_{G1-B4}$ may bind partially to non-cross-linked fibrin degradation products (FDPs), and AMBER$_{scFv-B4}$ favors cross-linked FDPs (xFDPs).
Extended Data Fig. 7 | Benchmarking of established inhibitors of coagulation. Benchmarking of established inhibitors of coagulation. Cells were incubated for 24 h in complete DMEM containing plasma or fibrinogen and either 1 µg/mL hirudin or 15 USP/mL heparin. Inhibition of coagulation by the thrombin inhibitors b) argatroban and c) ximelegatran was assessed by measuring the SEAP reporter production of cells expressing AMBERB4, AMBERB4/scFv or AMBERG1-B4. All values are means ± SD of n = 3 independent samples.
Extended Data Fig. 8 | Schematic illustration of vectors used for the generation of stable coagulation-sensing cells. Schematic illustration of vectors used for the generation of stable coagulation-sensing cells. Top: sensing and expression cassette for a secreted nano-luciferase (Nluc) reporter fused to a murine Fc tag for enhanced stability in vivo (Nluc-mFc) and a tenecteplase-mFc fusion construct (TNK-mFc) used in pTS992 (RE₄-P⁺CMV/lgkSS-Nluc-mFc₋RARYKR-TNK-mFc₋P⁺CMV-ZeoR-p2a-YPet-pA). Equimolar production and separation of the products during secretion are ensured by placing a furin cleavage site (RARYKR) between the two proteins. Middle: construct for stable integration of the reporter cassette using the Sleeping Beauty transposon system expressing a fluorescent protein (FP) to facilitate fluorescence-activated cell sorting (FACS) coupled to antibiotic resistance markers for zeocin (ZeoR) or blasticidin S (BlastR) for stable cell line selection. Bottom: schematic of constructs pTS941 (P⁺CMV-D-dimer-scFv-DARPinB4(tandem)-receptor-pA-P⁺SVA-STAT3-pA-P⁺CMV-TagBFP2-p2a-PuroR-pA) and pTS942 (P⁺CMV-RR120-scFv-receptor-pA-P⁺SVA-STAT3-pA-P⁺SVA-TagBFP2-p2a-PuroR-pA) for stable integration of the receptors as well as an overexpression module for signal transducer and activator of transcription 3 (STAT3) transcription factor. DR, direct repeat; Nluc, nano-luciferase; TNK, tenecteplase (htPA variant); mFc, murine antibody fragment for increased stability.
Extended Data Fig. 9 | Workflow to assess hirudin-HM2 efficacy in cell culture. Workflow to assess hirudin-HM2 efficacy in cell culture. mHEK_b4/scFv/hirudin cells were induced with xFDP protein on day 1. Supernatant of induced mHEK_b4/scFv/hirudin cells was mixed with 0.5% (v/v, final) plasma on day 2 and dispensed on coagulation-sensing cells expressing AMBER_b4. SEAP reporter expression from sensor cells and Nluc activity of mHEK_b4/scFv/hirudin are measured in the same sample with a multiplate reader on day 3.
Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐  The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

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Give P values as exact values whenever suitable.

☐  For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

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☐  Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Policy and code

Policy information about availability of computer code

Data collection  Microsoft Excel for Mac version 16.50

Data analysis  GraphPad Prism for MacOS version 9.1

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
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All data measured and analyzed in this study is available from the corresponding author on reasonable request.
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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size  No sample size calculation was done. Sample size (n=3 independent biological samples) was predicted to be sufficient to estimate the variation of the data and hence differences in means based on previous experiences on reporter gene expression.

Data exclusions  No data points were excluded.

Replication  All in vitro data was successfully replicated. Data from mouse experiments was not replicated due to sufficient group size and ethical reasons.

Randomization  No covariates due to sample allocation could be observed in cell culture and no randomization was performed. Experiments were done using the same mastermix to transfet all conditions of the same type. Treatments were also performed using the same solution to induce respective samples of mock/negative controls and functional receptor configurations. Differently transfected cells were all cultured in the same 96-well plate where the outer wells were left out to account for potential edge effects.

In mouse experiments, mice were randomly selected for each group.

Blinding  Cell culture experiments were not blinded since the use of multichannel pipetting and highly parallelized workflows for samples and controls reduce the likelihood to introduce even subconscious biases.
The blood sample analysis from mouse experiments was blinded.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a                              | n/a     |
| ☒ Involved in the study          | ☒ Involved in the study |
| ☐ Antibodies                     | ☐ ChiP-seq |
| ☒ Eukaryotic cell lines          | ☒ Flow cytometry |
| ☒ Palaeontology and archaeology  | ☒ MRI-based neuroimaging |
| ☐ Animals and other organisms    |         |
| ☐ Human research participants    |         |
| ☒ Clinical data                  |         |
| ☒ Dual use research of concern   |         |

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  HEK-293T (ACC 635) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ).

Authentication  Cells were recently obtained from the cell bank (DSMZ) or thawed from certified stocks and no further confirmation was performed.

Mycoplasma contamination  Cells were aquired mycoplasma free from ATCC and cultured under antibiotic free conditions, no test for mycoplasma contamination was performed.

Commonly misidentified lines (See ICLAC register)  Cells used are not listed in the ICLAC register.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  Wild-type male 6-week-old BABL/c mice were used in this study.
Wild animals
This study did not involve wild animals.

Field-collected samples
No samples were collected in the field.

Ethics oversight
The experiments involving animals were approved by the East China Normal University (ECNU) Animal Care and Use Committee and were in accordance with the Ministry of Science and Technology of the People’s Republic of China guidelines on animal care.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics
Blood samples were collected from routine blood testing at the hospital. Patients with DIC and regular patients were included. No additional information was obtained due to blinding.

Recruitment
Patient samples were collected from routine blood testing at the hospital. Samples showing normal and high D-dimer levels were selected.

Ethics oversight
Experiments involving patient samples were approved by the Medical Ethics Committee of Yangpu Hospital affiliated with Tongji University (request ID: LL-2018-SCI-003) and the Ethikkommission Nordwest- und Zentralschweiz (EKNZ) (request ID: 2021-01998).

Note that full information on the approval of the study protocol must also be provided in the manuscript.