

Target protein identification in live cells and organisms with a non-diffusive proximity tagging system

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The study presents **important** findings that reveal SEPHS2 and VPS37C as new potential drug targets for dasatinib and hydroxychloroquine respectively in addition to confirming known targets of these drugs. The evidence provided is **compelling** as observed in the methods, data and analyses. This article will be of great interest to chemical biologists, biochemists, and scientists in drug discovery and diagnostics.

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Abstract

Identifying target proteins for bioactive molecules is essential for understanding their mechanisms, developing improved derivatives, and minimizing off-target effects. Despite advances in target identification (target-ID) technologies, significant challenges remain, impeding drug development. Most target-ID methods use cell lysates, but maintaining an intact cellular context is vital for capturing specific drug-protein interactions, such as those with transient protein complexes and membrane-associated proteins. To address these limitations, we developed POST-IT (Pup-On-target for Small molecule Target Identification Technology), a non-diffusive proximity tagging system for live cells, orthogonal to the eukaryotic system. POST-IT utilizes an engineered fusion of proteasomal accessory factor A (PafA) and HaloTag to transfer Pup to proximal proteins upon directly binding to the small molecule. After significant optimization to eliminate self-pupylation and polypupylation, minimize depupylation, and optimize chemical linkers, POST-IT successfully identified known targets and discovered a new binder, SEPHS2, for dasatinib, and VPS37C as a new target for hydroxychloroquine, enhancing our understanding these drugs' mechanisms of action. Furthermore, we demonstrated the application of POST-IT in live zebrafish embryos, highlighting its potential for broad biological research and drug development.

Introduction

The landscape of modern pharmaceutical medicine has significantly evolved with the advent of enhanced drugs, targeted therapies, and combination treatments, yet variability in patient outcomes and toxic responses remain prevalent. This unpredictability stems from a complex interplay of factors, including incomplete target identification, unanticipated off-target effects, intricate polypharmacology, and the influence of individual genetics and environmental factors, all of which collectively contribute to the high failure rate of new drug candidates. This highlights the critical need for accurate identification of protein targets (target-ID). Phenotype- based drug screening offers a promising strategy for discovering novel or superior bioactive molecules, yet precise target-ID within this approach stands as a major barrier to advancing drug development further (Hughes et al., 2021 [DOI](#); Vincent et al., 2022 [DOI](#)). Target-ID is fundamental in unraveling the mechanistic pathways through which drugs exert their therapeutic effects, serving as a critical step in the refinement and development of targeted therapies. By enabling the precise tailoring of drugs to specific biological targets, target-ID significantly enhances drug specificity, efficacy, and safety, while also facilitating the repurposing of existing drugs and advancing our understanding of polypharmacology. Despite its critical importance, accurately identifying biological targets within the complex cellular environment of proteins and other molecules remains a challenge, often hindering progress from drug discovery to therapeutic application.

Significant advancements have been made in target-ID technologies, with each approach offering its own advantages and limitations (Ha et al., 2021 [DOI](#)). Affinity-based pull-down methods, traditionally employed for target-ID, are hindered by their dependence on strong binding interactions, potentially overlooking proteins that interact transiently or are present at low levels but are crucial for the drug's action. Additionally, affinity-based methods do not adequately preserve the intricate intracellular environment where these interactions occur. As an alternative, photo-affinity labeling (PAL) has emerged, utilizing analogs of bioactive molecules with photo-reactive groups to detect interactions in the natural cellular state. Despite its advantages, PAL has limitations such as nonspecific labeling, low photoreaction yields, orientation dependency, and in vivo incompatibility. In response, newer, modification-free approaches like stability of proteins from rates of oxidation (SPROX) (West et al., 2008 [DOI](#)), drug affinity responsive target stability (DARTS) (Lomenick et al., 2009 [DOI](#)), cellular thermal shift assay (CETSA) (Molina et al., 2013 [DOI](#)), and thermal proteome profiling (TPP) (Savitski et al., 2014 [DOI](#)) have been developed. These techniques rely on protein stabilization upon binding, offering benefits like avoiding the need for cumbersome synthesis of derivatives and preserving the cellular context (Molina et al., 2013 [DOI](#); Savitski et al., 2014 [DOI](#)). However, these methods may have difficulty detecting subtle or little changes in protein stability and have limited applicability to live cell conditions, underscoring the ongoing need for continued development in target identification methodologies.

Proximity labeling (PL) systems such as APEX (Martell et al., 2012 [DOI](#))/APEX2 (Lam et al., 2015 [DOI](#)), BioID (Roux et al., 2012 [DOI](#)), and TurboID (Branon et al., 2018 [DOI](#)) are rapidly emerging as effective tools for dissecting protein-protein or RNA-protein molecular interactions. While these methods offer significant advantages in mapping interactomes and networks, their diffusive labeling presents a limitation for accurate target-ID. Although certain developments such as the proximity-dependent target-ID system (PROCID) (Kwak et al., 2022 [DOI](#)) and biotin targeting chimera (BioTAC) (Tao et al., 2023 [DOI](#)) using TurboID and miniTurbo, respectively, have shown promise, the inherent diffusive nature still remains an obstacle to directly pinpointing target proteins, leading to increased potential for confusing results or necessitating extensive validation steps. Wells and colleagues developed a non-diffusive PL system for protein-protein interactions by fusing the E2-conjugating enzyme Ubc12 for NEDD8, a ubiquitin homolog, with a substrate binding domain/protein (Zhuang et al., 2013 [DOI](#)). This system was further adapted for target-ID by combining Ubc12 with a SNAP-tag for linkage to a specific small molecule, allowing Ubc12 to

covalently attach a biotinylated NEDD8 derivative to adjacent target proteins (Hill et al., 2016). While effective for target-ID, it has limitations. Exogenous expression of NEDD8 can lead to unintended labeling by the ubiquitination machinery (Enchev et al., 2015), thus restricting its application in live cells. Consequently, this system is generally utilized in vitro with cell lysates. While capable of tagging weakly interacting proteins, the system does not utilize the intact intracellular context, which is crucial for certain interactions. Additionally, the possibility of NEDD8 polyneddylation may introduce bias in labeling efficiency and affinity pulldown.

Furthermore, the typical neddylation conditions, occurring at 37 °C for 18 h, may cause protein instability and precipitation, although adjustments in incubation time or the addition of mild detergents may alleviate these concerns. Hence, there is a pressing need to develop innovative target-ID methods that enhance existing techniques.

To overcome existing limitations and establish a streamlined approach for target-ID in live cells and animal models such as zebrafish, we envisioned a non-diffusive PL system that leverages a prokaryotic ubiquitin-like protein, orthogonal to the eukaryotic ubiquitin proteasome system, thereby ensuring specificity and minimizing cross-reactivity. We chose the proteasomal accessory factor A (PafA) as a ligase and the prokaryotic ubiquitin-like protein (Pup) as its cognate substrate. Liu et al. had previously demonstrated its effectiveness as a PL system for studying protein-protein interactions in live cells (Liu et al., 2018). Here, we introduce a target-ID system, named Pup-On-target for Small molecule Target Identification Technology (POST-IT). This system integrates PafA with a HaloTag to attach a specific small molecule. After extensive optimization, we showed that POST-IT successfully identified many known targets and a novel binder, SEPHS2, for dasatinib in live cells. We then applied POST-IT for hydroxychloroquine (HCQ) and discovered VPS37C as a novel target, providing mechanistic insight into HCQ's action. Moreover, we have successfully demonstrated the application of POST-IT in live zebrafish embryos, highlighting its potential for broad usage in biological research.

Results

Design and optimization of POST-IT

To create a non-diffusive PL system suitable for universal and robust target-ID in live cells and animal models, we integrated PafA from *Corynebacterium glutamicum* (Cglu) as a non-diffusive PL effector with HaloTag as an anchor for small molecules of interest (Figure 1). PafA covalently transfers Pup to proximal prey proteins via direct contact on the ϵ -amino group of lysine residues (Özcelik et al., 2012; Watrous et al., 2010), a process known as pupylation. This approach offers an advantage due to PafA's inherent characteristic of promiscuous, low selectivity for the amino acid sequence (Liu et al., 2018; Watrous et al., 2010). We selected HaloTag over alternatives like SNAP-tag due to its superior live-cell labeling efficiency (Erdmann et al., 2019), readily accessible ligand surface (Kang et al., 2017; Los et al., 2008), and relatively simple chemical synthesis with a chloroalkane moiety (Los et al., 2008). To simplify our system for in vivo applications without requiring exogenous biotin, we fused Pup with either a streptavidin binding peptide (SBP) or a Strep-tag II (STII) as a handle to capture the targets, rather than using the commonly used biotin carboxyl carrier protein (BCCP). When a target protein binds to the compound linked to HaloTag, PafA is positioned in close proximity, allowing for the covalent attachment of Pup to the target's lysine residue. Pupylated targets can then be captured and identified by mass spectrometry.

To initiate the optimization of POST-IT, we reasoned that using smaller Pup substrates could minimize interference with the cellular functions of pupylated proteins. It has been shown that the N terminus of Pup can be removed without compromising its function, generating a short truncated protein with 28 amino acids from the C terminus (Liu et al., 2018). This truncated Pup

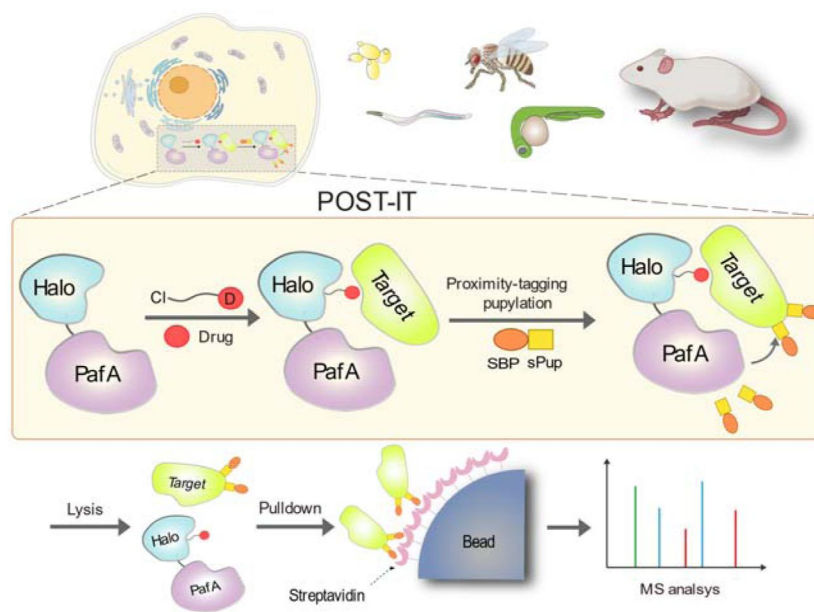


Figure 1.

Schematic illustration of the POST-IT system for target identification. Upon the introduction of a drug-specific HaloTag ligand derivative, POST-IT tags target proteins with sPup, a tagging process known as pupylation, in live cells or organisms. Subsequently, these proteins are enriched and identified via mass spectrometry (MS) analysis.

protein will be referred to hereafter as short Pup (sPup). We fused sPup with either an SBP or a twin-STII (TS) tag at its N terminus and used HB-Pup as a control, which contains 6×His and BCCP at the N terminus of Pup (**Figure 1**—figure supplement 1A). HaloTag was introduced at the N terminus of PafA. In vitro pupylation tests with recombinant proteins showed that the fusion of HaloTag did not impair PafA activity based on self-pupylation (**Figure 1**—figure supplement 1B). Notably, both types of sPup substrates exhibited similar pupylation levels to the full-length Pup. Additionally, PafA retained its robust activity even at 20 °C, a valuable feature for using this system in other animal models such as zebrafish and *C. elegans*, which naturally inhabit environments around this temperature. Moreover, we observed robust self-pupylation within 10 min at 37 °C. However, extensive incubation resulted in the formation of high molecular weight Halo-PafA, indicating polypupylation of Pup substrates (**Figure 1**—figure supplement 1C).

Polypupylation refers to the addition of a new Pup onto a previously linked Pup on the target protein, akin to polyubiquitination observed with ubiquitin. In contrast, multipupylation involves multiple single pupylations at different positions on the target proteins. Polypupylation can lead to unwanted biases in pupylation or pull-down efficiency, whereas multipupylation is more likely to correlate with binding affinity or interaction frequency. To counter polypupylation, we mutated lysine residues to arginine in TS-sPup and SBP-sPup (**Figure 1**—figure supplement 2A). Since the TS-tag contains two lysines, mutating sPup alone did not affect polypupylation (**Figure 1**—figure supplement 2B and C). However, further mutations of two lysine residues within the TS-tag, creating TS^{K8R}-sPup^{K61R}, completely abolished polypupylation in TS-sPup (**Figure 1**—figure supplement 2D). Interestingly, Halo-PafA with polypupylation branches, marked by yellow arrows, showed a distinct and slower migration pattern compared to multipupylation by TS^{K8R}-sPup^{K61R} at 0.5 h incubation. Similarly, modifying a lysine residue in the SBP-tag, either through mutation to SBP^{K4R} or by deleting the N-terminal four amino acids to create sSBP, also completely prevented polypupylation, as shown in sSBP-sPup^{K61R} and SBP^{4KR}-sPup^{K61R} (**Figure 1**—figure supplement 2E and F). Notably, we observed enhanced levels of multipupylation with lysine-removed sPup substrates over time (**Figure 1**—figure supplement 2D and F). Together, these results demonstrate that lysine to arginine (KR) mutation in sPup and modifications to the tags effectively eliminated polypupylation, while maintaining or enhancing the pupylation efficiency in a dose- and time-dependent manner.

In addition to polypupylation, we reasoned that the robust self-pupylation activity of Halo-PafA could diminish the pupylation of target proteins. To address this, we replaced all eight lysines with arginines in HaloTag, creating Halo^{8KR}-PafA (**Figure 1**—figure supplement 3A). Ts-sPup and TS-sPup^{K61R} were chosen as sPup substrates for this experiment, although any Pup substrates could have been used. The levels of self-pupylation were assessed. The result showed that Halo^{8KR}-PafA mutant exhibited a marked decrease in self-pupylation, similar to PafA alone, indicating inherent self-pupylation in PafA (**Figure 1**—figure supplement 3B). Previous research reported that PafA from *M. tuberculosis* (*Mtb*) possesses depupylase activity (Jiang et al., 2018), which may negatively impact pupylation levels of target proteins. Notably, an *Mtb* PafA mutant with a S119A mutation showed significantly reduced depupylase activity with minimal changes in pupylase activity (Jiang et al., 2018). Based on this, we hypothesized that inhibiting depupylation activity might enhance pupylation. Surprisingly, a PafA mutant with an S126A substitution (analogous to S119A in *Mtb* PafA) exhibited substantially increased pupylation activity with reduced polypupylation, as evidenced by the decreased levels of high molecular weight bands and an increase in low molecular weight bands (**Figure 1**—figure supplement 3A and B).

Validation of POST-IT with dasatinib in vitro

To assess the efficiency of our optimized Halo-PafA and Pup in pupylating target proteins for a small molecule, we synthesized DH1, a HaloTag ligand (HTL) derivative of dasatinib (**Figure 2A**), a well-characterized tyrosine kinase inhibitor used for leukemia treatment (Rix et al., 2007; Shi et al., 2012). Initially, we evaluated the functionality of Halo^{8KR} in Halo^{8KR}-PafA. Fluorescence polarization (FP) assay demonstrates the rapid binding of the Halo-AF488 ligand to Halo^{8KR}-PafA,

reaching a plateau in 15-20 min, consistent with a previous report (Los et al., 2008) (Figure 2—figure supplement 1A). A Halo-biotin competition assay confirmed further that DH1 could be efficiently linked to Halo^{8KR}-PafA within 15 min (Figure 2—figure supplement 1B and C).

After demonstrating the functionalities of Halo^{8KR} and DH1, we tested whether SRC, a known target of dasatinib, could be labeled by Halo-PafA in the presence of DH1. Surprisingly, robust pupylation of purified recombinant short SRC occurred exclusively with PafA harboring Halo^{8KR} at its N terminus in the presence of DH1, whereas PafA with Halo^{8KR} at the C terminus showed only modest labeling. This result highlights the importance of eliminating self-pupylation and the N-terminal positioning of HaloTag for effectively labeling the target protein (Figure 2B). We next examined the influence of KR mutations in sPup substrates. Both SBP^{K4R}-sPup^{K61R} and TS^{K8R}-sPup^{K61R} showed substantial levels of pupylation, especially at lower pupylation bands, suggesting increased multipupylation (Figure 2C). The pupylation of SRC by Halo^{8KR}-PafA with DH1 diminished with increasing concentrations of dasatinib, affirming the specificity of this system (Figure 2D). Notably, even with high concentration of SRC and Halo^{8KR}-PafA in the in vitro reaction, no pupylation was detected in the absence of DH1, indicating that the labeling by our PL system is non-diffusive, specific, and proximity-dependent.

Like SBP^{K4R}-sPup^{K61R}, we observed a significant increase in pupylation by PafA with the N-terminal, but not the C-terminal, Halo^{8KR} when using TS^{K8R}-sPup^{K61R} (Figure 2—figure supplement 2A) to a degree similar to that of SBP^{K4R}-sPup^{K61R} (Figure 2—figure supplement 2B). Although TS^{K8R}-sPup^{K61R} serves as an excellent substrate for PafA without causing polypupylation, the lysine mutations in the TS-tag led to a loss of binding to Strep-Tactin beads. Conversely, SBP^{K4R} and sSBP retained their binding to streptavidin, as the core binding sequence of SBP does not require the 1-10 amino acids at the N-terminus (Barrette-Ng et al., 2013).

Therefore, we decided to use the SBP-tag as a Pup substrate in the POST-IT system for further studies.

Validation of POST-IT with dasatinib in cellulo

Having successfully verified target protein pupylation by Halo-PafA in vitro, we evaluated our PL system under cellular conditions to minimize artifacts from non-physiological environments such as cell lysates. To mimic drug-target interaction-induced pupylation in live cells and assess the potential of PafA as a proximity-tagging system for target-ID, we incorporated the rapamycin-induced interaction between FRB and FKBP into our PL system, as this interaction between a small molecule and a protein is known to be highly specific and robust (Figure 3—figure supplement 1A). Rapamycin treatment led to robust pupylation of FKBP-EGFP by PafA fused with FRB in a Pup-dose dependent manner (Figure 3—figure supplement 1B and C), along with significant self-pupylation independent of rapamycin. Next, we investigated how KR mutations in SBP-sPup substrates affect pupylation efficiency in live cells. Both SBP-sPup and SBP^{K4R}-sPup^{K61R} exhibited efficient pupylation (Figure 3—figure supplement 1D), while sSBP-sPup^{K61R} failed to induce self-pupylation as well as pupylation of FKBP-EGFP, presumably due to its low expression or instability. Consequently, we chose SBP^{K4R}-sPup^{K61R} for subsequent studies to avoid polypupylation, although SBP-sPup did not cause any detectable polypupylation in this case. Interestingly, the presence of rapamycin greatly reduced self-pupylation when using either SBP^{K4R}-sPup^{K61R} or SBP-sPup, as shown in the lower panel of Figure 3—figure supplement 1D, suggesting that PafA constantly pupylates either itself or proximal target proteins. This underscores the importance of minimizing self-pupylation as a critical factor in developing for a better POST-IT system.

Given these findings, we sought to identify additional self-pupylation sites in PafA through mass analysis, uncovering six lysine residues susceptible to self-pupylation (Table 1). Focusing on the most frequently pupylated residue, we introduced an additional KR mutation at K172 (Figure 3—figure supplement 2), generating Halo^{8KR}-PafA^{S126A,K172R}.

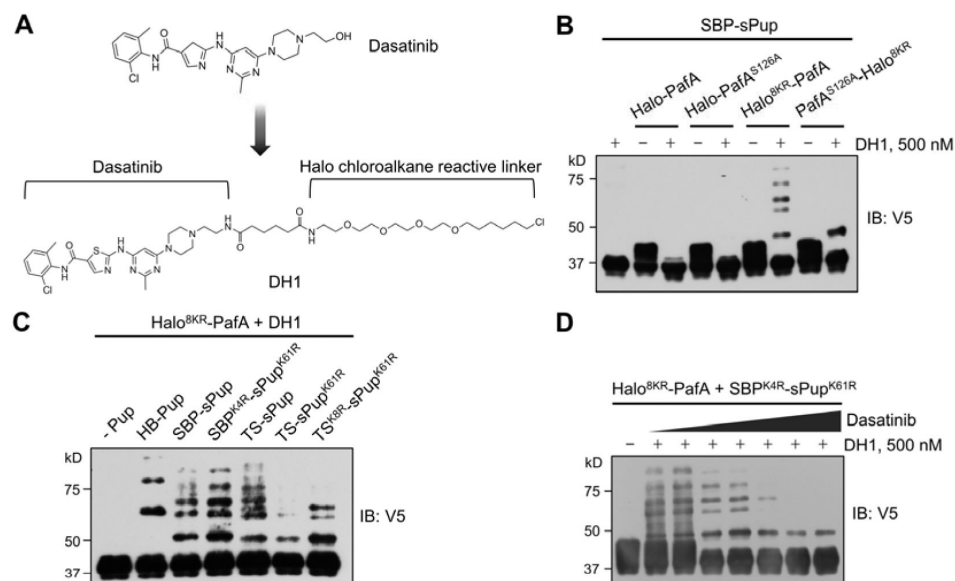


Figure 2.

Optimized Halo-PafA and Pup substrates efficiently promote pupylation in vitro in a proximity-dependent manner. **(A)** The chemical structure of DH1, an HTL derivative of dasatinib. **(B)** Comparison of proximity-tagging by different Halo-PafA derivatives on in vitro pupylation. **(C)** Comparison of different Pup substrates on in vitro pupylation with 500 nM DH1. **(D)** Pupylation levels by DH1 decrease with an increasing amount of dasatinib as a competitor, ranging from 0.2 μM to 10 μM . **(B-D)** All reactions were conducted with 1 μM of a Halo-PafA derivative, 10 μM of one of the different Pup substrates, and 0.5 μM of a purified short SRC with a 2 $\times$ V5 tag, SRC(247-536)-2 $\times$ V5, at 37 $^{\circ}\text{C}$ for 30 min. Pupylation levels of SRC were assessed by immunoblot (IB) using an anti-V5 antibody.

Figure 3.

POST-IT can label target proteins in live cells.

(A) Effect of linker length between Halo^{8KR} and PafA on target-labeling. HEK293T cells were co-transfected with HA-Halo^{8KR}-PafA containing the specified length of linker, SBP^{K4R}-sPup^{K61R}, and SRC(247-536)-2×V5. (B) Introducing mutations S126A and K172R into PafA significantly enhances its proximity-tagging efficiency on target. (C-E) Comparison of labeling activity of Halo-PafA and Halo^{8KR}-PafA^{S126A,K172R}, a form used for POST-IT. (F, G) POST-IT^{DH1} mediates proximity-tagging in a DH1-dose dependent manner (F), an effect completely inhibited by competitive dasatinib, ranging from 0.025 to 25.6 μM (G). Pupylation levels of exogenously expressed short SRC (C, F, G), endogenous SRC (D), or pull-downed endogenous SRC (E) were assessed by immunoblot (IB) analysis using antibodies against V5-tag or SRC for exogenous or endogenous SRC, respectively. In all experiments, SBP^{K4R}-sPup^{K61R} was used for co-transfection, and cells were treated with 500 nM DH1, except in (F, G).

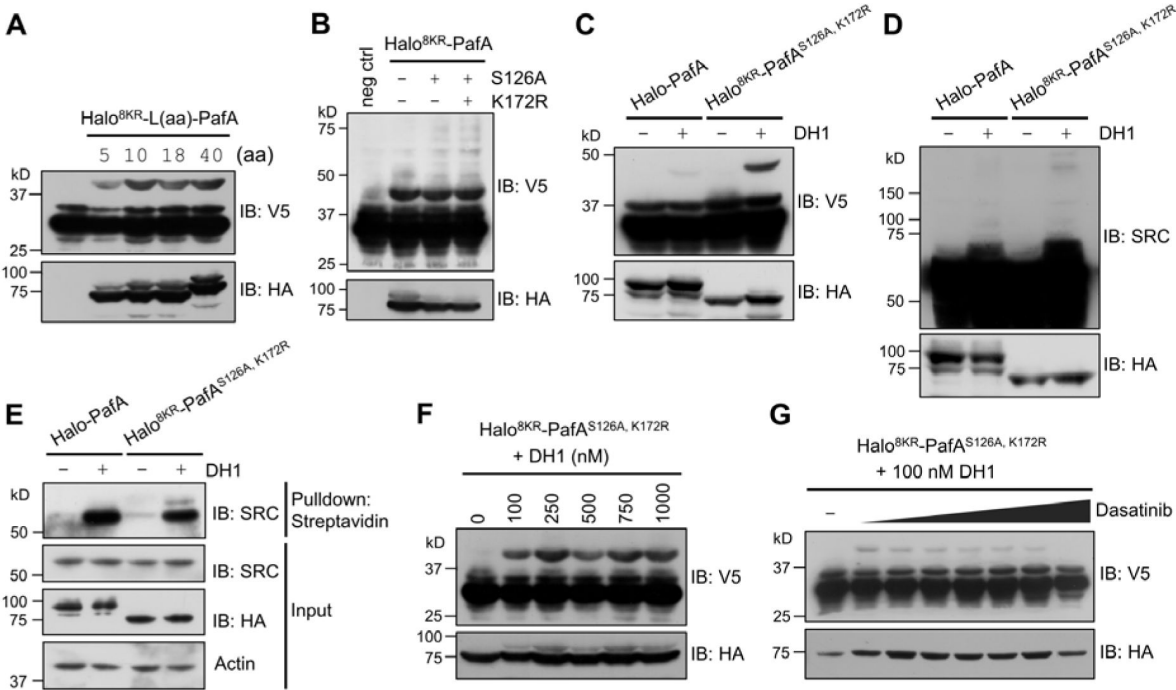


Table 1.

Identification of lysine residues for pupylation in PafA by mass spectrometry.

Protein	Score	Coverage (%)	# PSMs	Detected pupylated sequences	MH+ [Da]	Xcorr	Lysine	Rank of pupylation
PafA	607.055	89	1197					
			1	RIMGIEYGLTFVDGDSKK	2502.21808	5.40		
			2	RIMGIEYGLTFVDGDSKK	2518.21299	5.35	K29	3
			2	IMGIEYGLTFVDGDSKK	2362.11188	3.71		
			1	IMGIEYGLTFVDGDSKK	2346.11697	3.46	K30	5
			1	MFRPIVEKYSSNIFIPNGSR	2702.32428	2.93	K47	5
			1	MAVDAEESLAK	1406.64679	3.39		
			2	MAVDAEESLAKEDIAGQVYLFK	2670.29672	3.86	K106	2
			4	MAVDAEESLAKEDIAGQVYLFK	2686.29164	3.86		
			5	IHHNPPLDKGESFPLGYCISQR	2808.35222	5.10	K172	1
			3	IHHNPPLDKGESFPLGYCISQR	2809.33624	2.88		
			1	ILKAADTLGVPTVDWMR	2228.17436	3.61	K427	4
			1	ILKAADTLGVPTVDWMR	2244.16928	3.49		

To further refine our PL system, we tested the effect of linker length between Halo^{8KR} and PafA on SRC labeling in the presence of DH1. We observed enhanced labeling with a linker longer than 10 amino acids (**Figure 3A**), prompting us to select an 18-amino acid linker for further studies. We chose the 18-amino acid linker instead of the 40-amino acid linker for easier cloning and to lower the risk of DNA recombination from longer repeats. Additionally, a longer, flexible linker may behave like an intrinsically disordered protein (Harmon et al., 2017), which is an unwanted feature for target-ID. We then confirmed that the S126A and K172R mutations additively enhanced pupylation activity under cellular conditions, as the higher molecular weight multipupylation bands were slightly but noticeably increased with these mutations compared to Halo^{8KR}-PafA (**Figure 3B**). This optimized version of Halo-PafA, Halo^{8KR}-PafA^{S126A,K172R}, in combination with a pupylation-free version of sPup will be referred to as POST-IT. Unlike wild type Halo-PafA, which displayed negligible labeling under cellular conditions, POST-IT exhibited significant labeling activity on both exogenous and endogenous SRC in the presence of DH1, generating POST-IT^{DH1} (**Figure 3C-E**). Labeling intensity of short SRC increased with higher DH1 concentrations and was competitively inhibited by increasing concentrations of dasatinib (**Figure 3F and G**), demonstrating the specificity of POST-IT^{DH1} in target labeling.

In our quest for a superior PafA variant, we investigated the potential of *Mtb* PafA as a PL system. However, we detected no labeling of short SRC by *Mtb* PafA, irrespective of the positioning of Halo^{8KR} at the N- or C-terminus (**Figure 3**—figure supplement 3). In contrast, Halo^{8KR}-PafA (*Cglu*) exhibited significant pupylation with sPup^{K61R} substrates from both *Cglu* and *Mtb*, suggesting a lack of orthogonality among different prokaryotic PafA systems.

Optimization of the linker of dasatinib-HTL (DH) derivatives

Encouraged by the successful labeling of the target protein by POST-IT^{DH1} in live cells, we next explored its ability to identify known targets and potentially reveal novel binding proteins of dasatinib. To this end, we investigated whether the linker length or structure between dasatinib and the Halo chloroalkane moiety could influence labeling efficiency, as the linker's characteristics in biomolecules, including PROTAC (Békés et al., 2022), significantly impact their functionalities. Based on a previous report highlighting the enhanced performance of HTLs containing carbamate linkers (Friedman Ohana et al., 2015), we synthesized four additional DH derivatives (DH2-DH5) with varying linker lengths longer than that in DH1 (**Figure 4**—figure supplement 1). These new DH derivatives with carbamate linkers displayed significantly enhanced covalent binding to recombinant Halo^{8KR}-PafA in vitro and to transiently expressed Halo^{8KR}-PafA in intact cells, as measured by FP assays, compared to DH1 (**Figure 4A and B**). Importantly, the new DH derivatives also showed more robust pupylation of short SRC in vitro and endogenous SRC under cellular conditions (**Figure 4C and D**). DH5, containing the longest linker, was selected for further experiments due to its excellent efficiency. We then verified that POST-IT could efficiently pull down endogenous SRC in the presence of DH5, an effect attenuated by an excess of dasatinib (**Figure 4E**). Furthermore, we measured the inhibitory effect of DH5 on SRC kinase activity and confirmed that DH5 retains comparable activity to dasatinib (**Figure 4**—figure supplement 2).

Identification of target proteins for dasatinib by POST-IT

With our optimized POST-IT system and DH5, POST-IT^{DH5}, in hand, we conducted labeling experiments in live cells with or without an excess of dasatinib. After pulling down cell lysates with streptavidin beads, we analyzed biotin-eluted proteins via mass spectrometry. Remarkably, we identified 8 known target proteins (FYN, RIPK2, MAPK14, ABL2, ABL1, CSK, LYN, and SRC) with significant enrichment and 2 known target proteins (GAK and SIK2) with lower confidence (**Figure 5A**). Among these ten known targets, five are tyrosine kinases (FYN, ABL1/2, CSK, LYN, and SRC) and four are serine/threonine kinases (RIPK2, MAPK14, GAK, and SIK2). These results validate the effectiveness of our POST-IT system as a target-ID methodology and underscore its potential superiority to other PL systems for target-ID (Hill et al., 2016; Kwak et al., 2022).

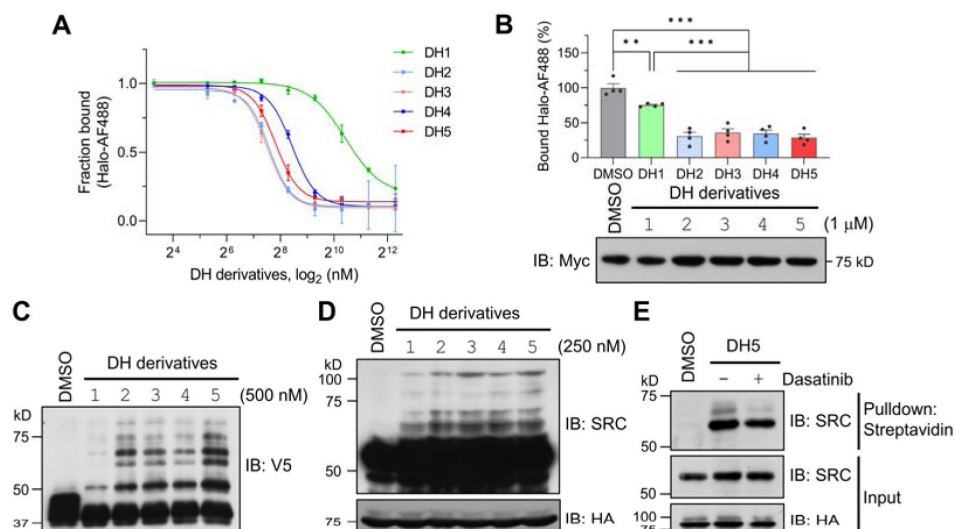


Figure 4.

Dasatinib-HTL (DH) derivatives with modified and longer linkers enhances POST-IT performance.

(**A**) In vitro binding assay for DH derivatives via fluorescence polarization (FP) assay. Purified Myc-Halo-PafA (15 nM) and 1 nM Halo-AF488 were incubated with various DH derivatives in a serial dilution at 37 °C for 30 min prior to FP measurement. Data are shown as mean ± s.d. (**B**) Cellular binding assay for DH derivatives by FP measurement. HEK293T cells transfected with Myc-Halo-PafA were later incubated with 1 μM of each DH derivative or DMSO for 3 h, followed by cell lysate incubation with 2 nM of Halo-AF488 at 37 °C for 30 min. *n* = 4. Data are shown as mean ± s.e.m. *P* values were calculated by an unpaired two-sided *t*-test. Immunoblot presents a representative image of the input levels for each condition, demonstrating that treatment with DH derivatives did not alter the expression levels of Myc-Halo-PafA. (**C**) In vitro labeling activity comparison among DH derivatives. Purified recombinant Halo^{8KR}-PafA (0.5 μM), SBP^{K4R}-sPup^{K61R} (10 μM), and SRC(247-536)-2×V5 (0.5 μM) were incubated with 0.5 μM of each DH derivative at 37 °C for 30 min. (**D**, **E**) In cellular labeling activity comparison among DH derivatives. HA-Halo^{8KR}-PafA and SBP^{K4R}-sPup^{K61R} were used for co-transfection, and cells were treated with 250 nM of various DH derivatives. Pupylation levels of purified short SRC (**C**), endogenous SRC (**D**), or pull-downed endogenous SRC (**E**) were assessed by immunoblot (IB) using anti-V5 or anti-SRC antibodies for exogenous or endogenous SRC, respectively.

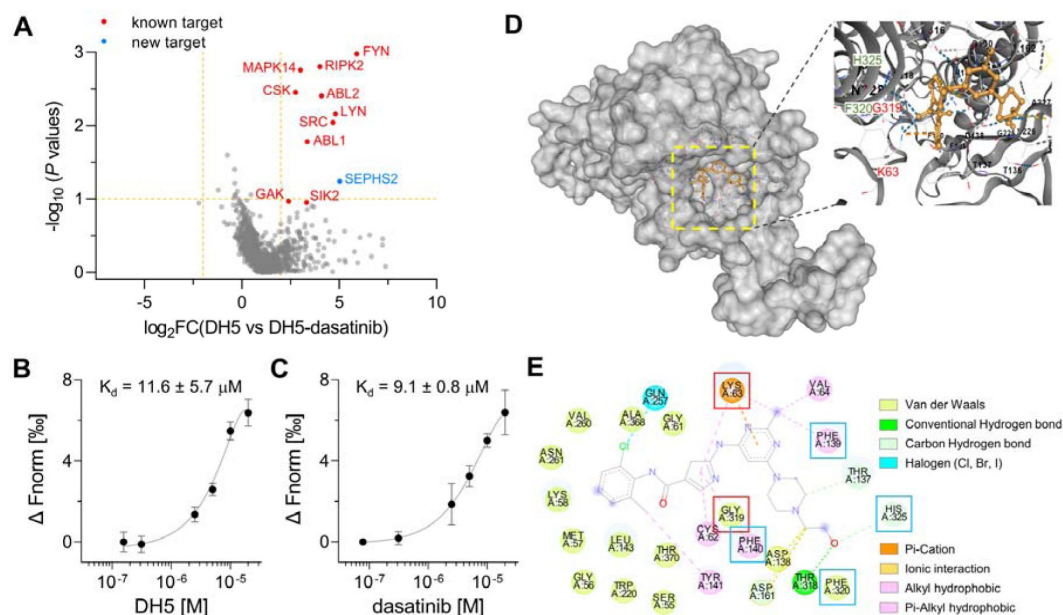


Figure 5.

Target-ID by POST-IT^{DH5}.

(A) Volcano plot displaying the relative fold change (FC) of DH5-binding proteins compared to those with dasatinib competition. Known target proteins for dasatinib were highlighted in red, and a newly identified target protein, SEPHS2, was marked in blue. (B, C) MST analysis demonstrates a direct interaction between DH5 and Cy5-labeled SEPHS2 (B) or between dasatinib and Cy5-labeled SEPHS2 (C), yielding K_d values of $11.6 \pm 5.7 \mu M$ and $9.1 \pm 0.8 \mu M$, respectively. Data are shown as mean $\pm$ s.d. (D) Molecular docking binding pose between dasatinib and SEPHS2. The yellow box indicates the binding pocket, expanded for a closer view. The binding pocket of dasatinib precisely fits into the active sites of SEPHS2. (E) Diagram of 2D molecular docking interaction between SEPHS2 and dasatinib. Red boxes highlight the active sites, and blue boxes indicate residues among HUB nodes.

Additionally, we identified selenophosphate synthetase 2 (SEPHS2) as a novel protein target that binds to dasatinib. SEPHS2, a crucial enzyme for selenoprotein synthesis, exhibits ATP- binding and kinase activities, supporting its potential interaction with dasatinib. To confirm SEPHS2 as a genuine target of dasatinib, we purified the recombinant SEPHS2 protein and measured its binding affinity to DH5 using microscale thermophoresis (MST) analysis (**Figure 5B and C**). This analysis yielded a dissociation constant (K_d) of $11.6 \pm 5.7 \mu\text{M}$ for DH5 and $9.1 \pm 0.8 \mu\text{M}$ for dasatinib. To elucidate the binding mode of dasatinib to SEPHS2, we performed molecular docking analysis to model their interaction. A previous study using molecular dynamics simulations identified Sec60, Lys63, and Gly319 as active sites and 15 amino acids as key residues or HUB nodes (Nunziata et al., 2019). Remarkably, the docking analysis revealed a favorable binding mode for dasatinib within the active sites of SEPHS2, with a Vina score of - 9.9 (**Figure 5D**). In the binding pocket of SEPHS2, dasatinib engages in multiple interactions, including pi-cation or cation- π interaction with Lys63 and Van der Waals interaction with Gly319 in the active sites, along with hydrophobic interactions with Phe130 and Phe140, Van der Waals interaction with Phe320, and a hydrogen bond with His325 among the HUB nodes (**Figure 5E**). Collectively, these results suggest that SEPHS2 is a novel protein target for dasatinib, indicating that dasatinib may impact SEPHS2 function.

Identification of target proteins for chloroquine by POST-IT

To further demonstrate the effectiveness of the POST-IT system in target-ID, we applied it to identify target proteins for hydroxychloroquine (HCQ), using it as an additional model ligand. HCQ has a long history of clinical use for treating malaria, rheumatoid arthritis, and lupus.

Moreover, it has been widely used in research as an autophagy inhibitor, a property gaining interest in the development of cancer therapy (Jain et al., 2023). Despite its broad application, the precise mechanisms through which HCQ inhibits autophagy and the pathways through which HCQ or chloroquine (CQ) elicit adverse effects such as psychiatric symptoms, retinal and ototoxicity, cardiac toxicity, and even death (Muller, 2021; Nirk et al., 2020) remain to be fully understood.

First, we synthesized a dimer form, DC661-H1, of a CQ HTL derivative with the same linker used for DH5 (**Figure 6**—figure supplement 1A), assuming that a dimer would enhance binding affinity as previously described (Rebecca et al., 2019). We confirmed that DC661-H1 and DC660, an analog of DC661-H1 without HTL, induced robust LC3-II bands in two mammalian cell lines to a similar extent as HCQ, indicating that they function as potent autophagy inhibitors (**Figure 6**—figure supplement 1B). To test the versatility of POST-IT as a tool for mass analysis, POST-IT was coupled with stable isotope labeling with amino acid in cell culture (SILAC) for target-ID. Heavy-Lys/Arg-labeled HEK293T cells expressing POST-IT were treated with DC661-H1 and DMSO, while light-Lys/Arg-labeled HEK293T cells expressing POST-IT were treated with DC661-H1 and an excess of competitive DC660, as described in **Figure 6**—figure supplement 2. Intriguingly, POST-IT identified several target proteins directly linked to autophagy regulation (**Figure 6A**). Hence, we aimed to verify whether DC660 and HCQ bind to these autophagy-related proteins. Among them, we purified five recombinant proteins, including TOM1, TOM1L2, TRAPPC3, VPS29, and VPS37C, and measured their binding properties using MST analysis. TOM1, TOM1L2, and TRAPPC3 appeared to bind well to DC660 but failed to acquire K_d values due to abnormal thermophoretic movement, suggesting non-homogeneous aggregation (**Figure 6**—figure supplement 3A-C). VPS29 showed dose-dependent binding to DC660, yielding a K_d of $24.95 \pm 11.71 \mu\text{M}$ (**Figure 6**—figure supplement 3D). However, binding to HCQ was negligible in these proteins (**Figure 6**—figure supplement 3E-H). These results support the validity of our POST-IT as a PL system for live cell target-ID, as all tested proteins showed binding ability to DC660.

Next, we investigated the binding affinity of VPS37C to DC660 and HCQ using purified recombinant protein. MST analysis revealed a very high binding affinity of VPS37C to DC660 with a K_d value of $21.5 \pm 9.8 \text{ nM}$ (**Figure 6B**). Notably, VPS37C also exhibited moderate binding affinity

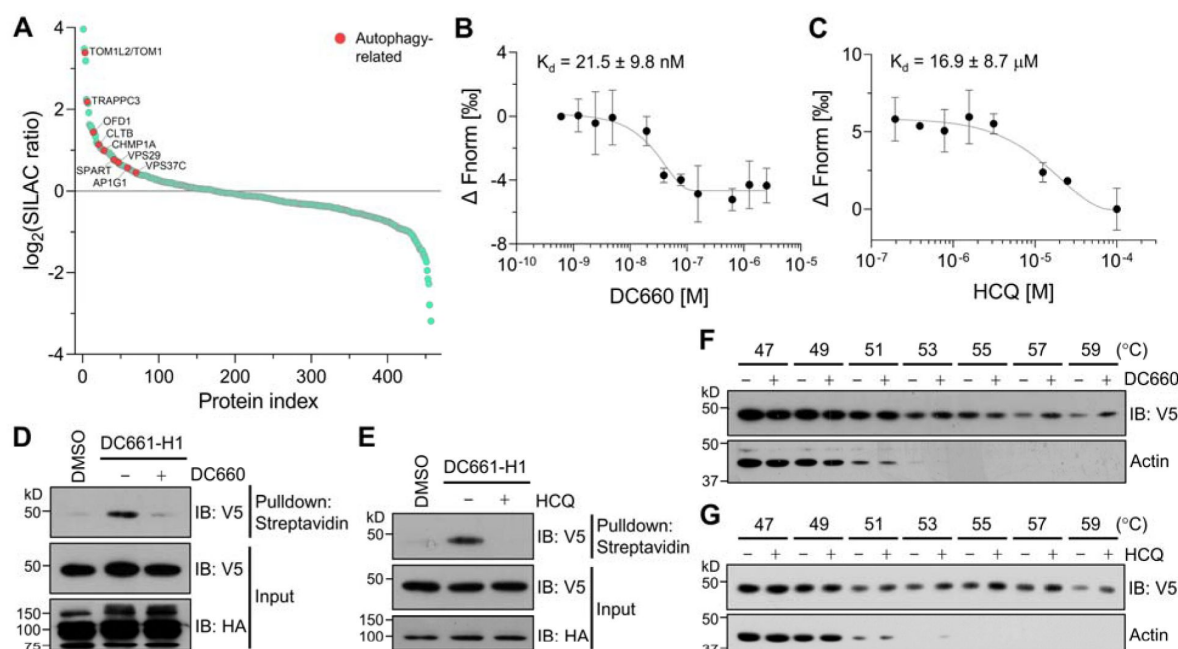


Figure 6.

Target-ID for HCQ by POST-IT^{DC661-H1}.

(**A**) Rank plot analysis of SILAC ratio values of proteins in three biological replicates. Autophagy-related proteins ranked highly are marked in red. Heavy-labeled cells were treated with 200 nM DC661-H1, whereas light-labeled cells underwent incubation with both DC661-H1 (200 nM) and competitive DC660 (2 μM). (**B**, **C**) MST analyses demonstrate direct interactions of DC660 (**B**) and HCQ (**C**) with VPS37C, yielding K_d values of 21.5 ± 9.8 nM and 16.9 ± 8.7 μM, respectively. Data are shown as mean $\pm$ s.d. (**D**, **E**) Immunoblot results indicate that the VPS37C-V5 protein from cells treated with DC661-H1 was significantly enriched after streptavidin pulldown. The competition between DC661-H1 and either DC660 (**D**) or HCQ (**E**) nearly completely abolished VPS37C binding. HEK293T cells were co-transfected with HA- Halo^{8KR}-PafA^{S126A,K172R}, SBp^{K4R}-sPup^{K61R}, and VPS37C-V5. After 24 h, cells were incubated with 200 nM of DC661-H1, with or without 2 μM of DC660 (**D**) or HCQ (**E**). Twenty-four hours later, cells were collected for further analysis. (**F**, **G**) CETSA results demonstrate that VPS37C becomes thermostable when exposed to DC660 (**F**) or HCQ (**G**). Selective stabilization of VPS37C is evident at temperatures of 53 °C or higher, a phenomenon not observed in β -actin.

to HCQ (**Figure 6C**). This result prompted us to further explore the binding ability of VPS37C under cellular conditions. First, we performed POST-IT labeling in HEK293T cells expressing VPS37C with a V5-tag, followed by pulldown using streptavidin beads.

VPS37C was substantially enriched in the presence of DC661-H1, but its binding was almost completely abolished by competitive DC660 or HCQ (**Figure 6D and E**). Furthermore, through a CETSA experiment, we observed that VPS37C became more stable in the presence of DC660 or HCQ than in the DMSO condition, upon exposure to increasing temperatures from cell lysates (**Figure 6F and G**). Collectively, these results provide strong evidence that VPS37C binds to DC661 (or DC660) as well as HCQ in vitro and in live cells, suggesting a potential role of VPS37C in the action mechanism of CQ or HCQ in inhibiting autophagy.

In vivo application of POST-IT as a target-ID system

Phenotype-based screening is gaining increased recognition as a powerful strategy for discovering novel compounds, superior drugs, or uncovering unknown biological mechanisms, such as the non-canonical translation discovered in our previous study using this approach (Jin et al., 2018). Zebrafish have emerged as an excellent in vivo whole-animal model system for small molecule screening and drug discovery. They are vertebrates, highly prolific, and amenable to high-throughput chemical screens, often leading to the serendipitous discovery of novel compounds (Patton et al., 2021). However, target-ID remains a major hurdle in understanding the mechanisms of action of novel compounds, thereby delaying the advancements in drug development. Therefore, we set out to test the applicability of POST-IT as an efficient method for target-ID in zebrafish.

To this end, we first generated a robust, ubiquitous expression plasmid for POST-IT in zebrafish using an optimized QF-binary system (Burgess et al., 2020). This system combines a DNA binding domain from the QF transactivator fused with an activation domain from the Gal4 transactivator, creating QFGal4, which binds to five repeats of QUAS, leading to gene activation. POST-IT, tagged with a 3×FLAG, is inserted following a 2A ribosome-skipping sequence, allowing for the simultaneous expression of QFGal4 and POST-IT under the control of a ubiquitous promoter, *ubb* (**Figure 7A**). We chose the *ubb* promoter over tissue-specific promoters to more accurately evaluate the levels and potential toxicity of transgene expression.

The expression of SBP^{K4R}-sPup^{K61R} is driven by a 5×QUAS promoter, and EGFP is incorporated upstream of a 2A sequence as an expression marker. All these components are assembled in a single plasmid to simplify transgenesis. Embryos injected with this plasmid displayed robust EGFP expression throughout their entire bodies without observable toxicity, indicating the suitability of the POST-IT system in zebrafish (**Figure 7B**; **Figure 7C**—figure supplement 1A). In addition, we generated a transgenic zebrafish line that expresses POST-IT under a heat-shock promoter. Embryos from this transgenic line show no obvious toxicity after heat shock (**Figure 7C**—figure supplement 1B and C). Note that the expression pattern of EGFP is non-homogeneous due to the mosaic expression of the injected plasmid. After treating live embryos with DH5 or DMSO, we observed marked enrichment of endogenous SRC in embryos treated with DH5 but not in those treated with DMSO, although expression levels of POST-IT were slightly lower in the DH5 group due to injection variability (**Figure 7C**). Together, these results demonstrated that POST-IT can be effectively utilized as a transgene in zebrafish without toxicity and applied for in vivo target-ID.

Discussion

In this study, we present POST-IT (Pup-On-target for Small molecule Target Identification Technology), a new non-diffusive PL system for target-ID in live cells and in vivo animal models. We systematically employed mutagenesis to eliminate self-pupylation, polypupylation, and

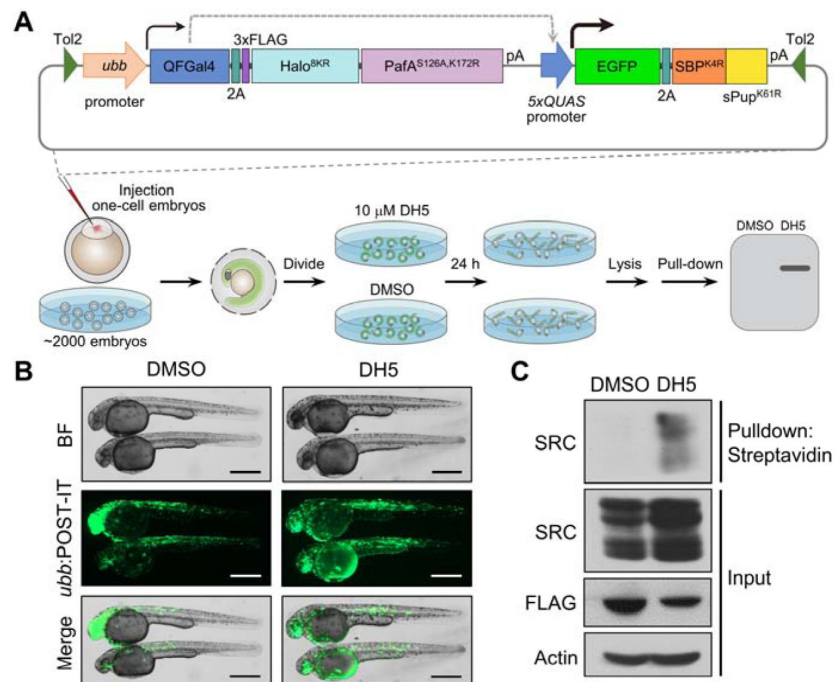


Figure 7.

POST-IT enables in vivo target-ID.

(A) Schematic of experimental design for applying POST-IT in zebrafish. A plasmid construct containing the POST-IT system was injected into embryos at the one-cell stage. The injected embryos were dechorionated 24 h later, and then treated with DH5 or DMSO for an additional 24 h. Embryos lysates were collected and analyzed by immunoblot. (B) Representative images of injected embryos. Bright field (BF) images reveal no overt toxicity from POST-IT expression. EGFP images display robust expression of POST-IT. Scale bar, 500 μ m. (C) Immunoblot analysis demonstrates that POST-IT exhibits significant enrichment of SRC in the presence of DH5 but not DMSO.

depupylase activity. Moreover, we optimized the linker length between HaloTag and PafA and developed PEG linkers for HTL derivatives. POST-IT provides a straightforward, yet highly robust and reliable method for target-ID within intact cellular context, a feature that significantly reduces artifacts from cell lysates and may be crucial for certain ligand/drug-protein interactions. We further demonstrated the applicability of POST-IT in an animal model, zebrafish. POST-IT does not cause noticeable toxicity and allows for tissue-specific and temporally controlled expression using a variety of transgenic zebrafish systems. We believe that POST-IT can be readily applied to other organisms, from *C. elegans* to mice, while retaining its effectiveness.

This is evidenced by the robust activity of Halo-PafA, which remains active even at temperatures as low as 20 °C (**Figure 1** [↗](#)—figure supplement 1B).

While TurboID is a widely used tool for studying protein networks, it suffers from persistent high background labeling, even without biotin. This necessitates meticulous experimental adjustments, such as employing dialyzed FBS and fine-tuning biotin labeling levels (May et al., 2020 [↗](#)). Alternative strategies, such as light-regulation (Lee et al., 2023 [↗](#)) and split-TurboID (Cho et al., 2020 [↗](#)), have been introduced to mitigate high background labeling, yet their suitability for target-ID and in vivo applications remains to be explored. Additionally, previous studies have reported cellular toxicity associated with TurboID (Branon et al., 2018 [↗](#); May et al., 2020 [↗](#)), although recent work shows no obvious toxicity in zebrafish (Xiong et al., 2021 [↗](#)). Furthermore, the labeling radius of TurboID can exceed 35 nm (May et al., 2020 [↗](#)), complicating the precise identification of target proteins. While these PL systems can provide valuable insights into a drug's protein network (Kwak et al., 2022 [↗](#); Tao et al., 2023 [↗](#)), direct target-ID is essential for understanding the drug's mechanism of action.

A recent study introduced BmTyr, a PL system based on *Bacillus megaterium* tyrosinase (Zhu et al., 2024 [↗](#)), which oxidizes phenol or catechol derivatives into *o*-quinones for labeling adjacent proteins. This method provides fast and lower-background labeling compared to TurboID. Unlike APEX, BmTyr does not require hydrogen peroxide, enhancing its biocompatibility for in vivo use. However, the need for copper might introduce toxicity and limitations. Oxygen dependency and glutathione reactivity may also affect its performance. While in vivo application was demonstrated by injecting purified BmTyr into mouse brains, the feasibility of its transgenic expression and broader application remains untested. Importantly, the diffusive nature of BmTyr's labeling might challenge its use for precise target-ID, despite being an innovative alternative PL toolkit.

Using our POST-IT system, we identified SEPHS2 and VPS37C as novel target proteins for dasatinib and HCQ/CQ, respectively. SEPHS2 detoxifies selenide, a toxic intermediate of selenocysteine biosynthesis, by converting it with ATP into selenophosphate, an essential component for selenoprotein production. This function is particularly important in cancer cells that require enhanced defense against oxidative stress. These cancer cells frequently exhibit elevated SLC7A11/xCT expression, leading to increased selenide import and a heightened demand for selenide detoxification. Similarly, abnormal upregulation of selenium uptake and selenocysteine biosynthesis occurs in many cancer cells, including breast cancers (Carlisle et al., 2020 [↗](#)) and acute myeloid leukemia (Eagle et al., 2022 [↗](#)), making SEPHS2 crucial for their survival but not for normal cells. Therefore, SEPHS2 is a promising target for cancer therapy. Given that SEPHS2 has been identified as a target for dasatinib, potentially binding to the active site, investigating whether SEPHS2 inhibition by dasatinib contributes to its anticancer effects is warranted. Binding information of dasatinib to SEPHS2 could facilitate the development of new SEPHS2 inhibitors.

Despite decades of research using HCQ/CQ as an autophagy inhibitor, its exact mechanism of action remains elusive. Although it has been widely assumed that HCQ/CQ increases lysosomal pH, Mauthe et al. demonstrated that its primary effect is to inhibit the fusion between autophagosomes and lysosomes (Mauthe et al., 2018 [↗](#)). Additionally, HCQ/CQ induces

disorganization in the Golgi and endosomal systems independently of autophagy. These findings highlight the significance of pH-independent mechanisms in HCQ/CQ's action. Furthermore, components of the Endosomal Sorting Complex Required for Transport (ESCRT) complexes have been shown to regulate the final stages of autophagosome formation (Javed et al., 2023 [↗](#); Takahashi et al., 2019 [↗](#), 2018 [↗](#); Zhen et al., 2020 [↗](#)). ESCRT-I consists of three core components (TSG101/VPS23, VPS28, and one of VPS37A/B/C/D) and a single auxiliary protein among UBAP1, MVB12A, and MVB12B. While VPS37A has been identified as a key player in phagophore closure (Javed et al., 2023 [↗](#); Takahashi et al., 2019 [↗](#)), the roles of other VPS37 proteins remain unexplored. Considering the structural similarities among the VPS37 proteins (**Figure 6** [↗](#)—figure supplement 4), it is plausible that HCQ/CQ could interact with multiple members of this family to some extent, potentially inhibiting their role in autophagosome maturation. This suggests two potential mechanisms for HCQ/CQ-mediated autophagy inhibition. One involves interfering with the function of VPS37 proteins in autophagosome closure. The other suggests that HCQ/CQ is actively transported into lysosomes by ESCRT-I, leading to lysosomal alkalization. Further research is crucial to elucidate these mechanisms. Understanding these pathways will not only illuminate therapeutic strategies for HCQ/CQ but also guide the development of superior derivatives with minimized side effects.

There remain some limitations in utilizing POST-IT system. One limitation is the requirement to synthesize an HTL derivative for the compound of interest, a challenge common to all target-ID methods except for those that are modification-free. To facilitate derivatization, we provide guidelines for linkers incorporating carbamate and PEG, as shown in **Figure 4** [↗](#) and **Figure 4** [↗](#)—figure supplement 1. PEG linkers are advantageous for their increased solubility and cell permeability. Provided the derivatives maintain comparable binding affinity to the target protein or preserve the compound's activity, we believe that POST-IT can be effectively applied from in vitro conditions to live cells and organisms. Secondly, POST-IT may require significantly longer incubation times in live cells compared to in vitro reactions, which could be attributed to several factors, such as insufficient expression of POST-IT or Pup, or the instability or metabolism of an HTL derivative of the drug. One potential approach to improve reproducibility is to generate stable cell lines and transgenic zebrafish lines that express high levels of POST-IT, which is feasible due to the lack of observable toxicity from POST-IT expression. High levels of Pup expression are especially important for efficient tagging.

Establishing stable cell lines with robust POST-IT expression could also enhance scalability for drug discovery applications. Additionally, further improvements of POST-IT through directed evolution, rational protein engineering, and structure-aided design are warranted. Studying the metabolism, stability, and toxicity of HTL derivatives of the drug is also valuable for better experimental design. Given that many drugs function in specific cellular locations, such as the cell surface and mitochondria, targeting POST-IT to precise subcellular locations could enhance the likelihood of identifying true biological target proteins rather than off-target binding partners. Therefore, exploring the functionalities of POST-IT in specific subcellular contexts is an essential direction for future research. Additionally, in this study, we utilized HEK293T cells for proof-of-concept. However, investigating protein targets in diverse cell types, particularly cancer cells, may reveal a broader spectrum of targets. To gain a more comprehensive understanding of a drug's target profile, we recommend that users employ POST-IT in various cell lines or in vivo models, such as zebrafish.

In conclusion, POST-IT represents a significant advancement in the field of target identification, offering an innovative, non-diffusive proximity tagging system designed for precise target-ID within live cells and in vivo models. By preserving the cellular context, POST-IT overcomes the limitations associated with traditional cell lysate methods and offers superior specificity compared to existing PL systems like TurboID. We successfully identified SEPHS2 as a target for dasatinib and VPS37C as a target for HCQ, exemplifying its effectiveness. Its application in both live cells and

zebrafish embryos underscores its versatility and potential to provide valuable insights into the molecular mechanisms of drug action, thereby enhancing the scope of biomedical research and therapeutic development.

Materials and methods

Materials

General reagents and solvents including NaCl, KCl, EDTA·Na₂, KH₂PO₄, Na₂HPO₄·12H₂O, NaF, NH₄OH, NaOH, urea, chloroform, glycerol, methanol, ethanol, isopropanol, and others, were obtained from Sinopharm Chemical Reagent, China. Regents related to protein expression, such as Isopropyl β-D-1-thiogalactopyranoside (IPTG), Tricine, sodium dodecyl sulfate (SDS), acrylamide, N,N'-methylene-bis-acrylamide, tetramethylethylenediamine (TEMED), ammonium persulfate, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), imidazole, Tris-HCl, glycine, ATP·Na₂, and biotin, were obtained from aladdin or Biofroxx, China. Triton X-100 and Tween-20 were from Sangon Biotech, China, and NP-40 was from Biosharp, China. Ni Sepharose 6 Fast Flow (17531801) was from Cytiva, USA, glutathione resin (SA008010) was from Smart-Lifesciences, China, and Sephadex-G100 (BS210) was from Biosharp, China.

Amicon Ultra Centrifugal Filters (UFC9050, UFC9030, UFC9010) were purchased from Millipore, USA. For molecular cloning, 2×MultiF Seamless Assembly Mix (RK21020) was from ABclonal, China, and Phanta Max Super-Fidelity DNA Polymerase (P505-d1) and HiScript 1st strand cDNA Synthesis Kit (R312) were from Vazyme, China. All restriction enzymes were from New England Biolabs, USA. HaloTag Alexa Fluor 488 ligand (G1001) and HaloTag Biotin ligand (G8281) were from Promega, USA. Dasatinib (S45672-25MG) was purchased from Shanghai yuanye Bio-Technology, China. Hydroxychloroquine (HCQ) (T9287) was obtained from TargetMol Chemicals, USA. BCA assay (P0011) was from Beyotime, China. Clarity Western ECL (1705060) was purchased from Bio-Rad, USA. Antibodies against V5-tag (AE017), Flag-tag (AE092), HA-tag (AE008), Myc-tag (AE070), and β-actin (AC026), as well as secondary antibodies, were purchased from ABclonal, China. Anti-SRC (36D10) (2109) and Streptavidin-HRP (3999) were from Cell Signaling Technology, USA. Streptavidin magnetic beads (L-1012) was purchased from BioLinkedin, China. Information on all other materials is provided in the methods section as they appear.

Construction of plasmids

Plasmids were typically constructed by the HiFi DNA assembly method using 2×MultiF Seamless Assembly Mix. PCR amplification was carried out with a high-fidelity DNA polymerase, Phanta Max Super-Fidelity DNA Polymerase. In general, the pTol2-EGFP-2ApA-CK2 or pTol2-EGFPpA vector, developed in our lab (Jin et al., 2018 [\[18\]](#)), was used for mammalian expression, and the pET28a vector was utilized for recombinant protein expression in *E. coli*, unless specified otherwise. All DNA constructs were validated by Sanger sequencing.

To construct Halo-PafA plasmids, HaloTag and PafA were PCR amplified using HaloTag7 (a gift from Yang Yu, Chinese Academy of Sciences) and pEF6a-kozak-CD28-PafA-myc (a gift from Min Zhuang, ShanghaiTech University), respectively, and then subcloned into the NotI and EcoRI sites of pTol2-EGFP-2ApA-CK2, or into the EcoRI site of pET28a. HaloTag^{8KR}, TS-sPup, and SBP-sPup DNA fragments were synthesized by Tsingke Biotechnology (Wuhan).

Halo^{8KR}-PafA was subcloned into the NotI and EcoRI sites of pTol2-EGFP-pA or into the BamHI and NotI sites of pET28a. TS-sPup and SBP-sPup were subcloned into the KpnI and NotI sites of pEF6a-HB-PUP (a gift from Min Zhuang) for mammalian expression and inserted into the EcoRI site of pET28a for *E. coli* expression. Point mutations, including SBP^{K4R}, TS^{K8R}, sPup^{K61R}, PafA^{S126A}, and

PafA^{K172R}, were introduced using a modified site-directed mutagenesis (Liu and Naismith, 2008 [\[1\]](#)). For recombinant protein expression of PafA in *E. coli*, PCR-amplified PafA was fused to a GST-tag into the BamHI and EcoRI sites of pGEX-6P-2, generating pGEX-6P-2-PafA.

Target proteins, such as SRC, SEPHS2, VPS37C, TOM1, TOM1L2, TRAPPC3, and VPS29, were PCR amplified using a cDNA library synthesized with the HiScript 1st strand cDNA Synthesis Kit from total RNA extracted from HEK293T cells with RNAiso Plus (9109, Takara Bio, Japan). A catalytic active, truncated version of SRC spanning amino acids 247-536, SRC(247-536), was inserted into the NotI and EcoRI sites of pTol2-EGFP-2ApA-CK2, and into the BamHI and NotI sites of pET28a. SEPHS2-2×V5 was subcloned into the BamHI and EcoRI sites of pET28a-N-SBP, engineered by inserting a SBP-tag into pET28a vector in our lab for *E. coli* expression. The selenocysteine (Sec60) in SEPHS2 was mutated to cysteine for expression in *E. coli*. VPS37C-2×V5 was subcloned into the NotI and MluI sites of pHAGE-IRES-puro (a gift from Lingling Chen, ShanghaiTech University) for mammalian expression, and the codon-optimized VPS37C, coVPS37C, for *E. coli* was inserted into the EcoRI and SalI sites of pET28a-N-SBP for *E. coli* expression. TOM1, TOM1L2, TRAPPC3, and VPS29 were subcloned into the EcoRI site of pET28a.

FKBP and FRB were synthesized by Tsingke Biotechnology (Wuhan). FKBP, tagged with a 3×HA, was inserted into the NotI and EcoRI sites of pTol2-EGFPpA, generating pTol2-3×HA-FKBP-EGFPpA. FRB, tagged a 3×V5, and mKate2 were assembled into the NotI and XmaI sites of pTol2-Myc-Halo-PafA-2ApA-CK2, resulting in pTol2-3×V5-FRB-mKate2-PafA-pA. To make a stable cell line, CMV-HA-Halo^{8KR}-PafA^{S126A,K172R}, SBP^{K4R}-sPup^{K61R}, TRE3G (a Tet-On promoter), Tet-On 3G transactivator, IRES-EGFP, and Puro were PCR amplified and assembled into pPB-mU6pro (a gift from Zhou Yan, Wuhan University), generating PB-puro-CMV-POST-IT-Tet-SBP^{K4R}-sPup^{K61R}-iresGFP. The expression of SBP^{K4R}-sPup^{K61R} was induced by the application of doxycycline via the Tet-On promoter. The P2A, a 2A ribosome-skipping sequence, was placed between HA-Halo^{8KR}-PafA^{S126A,K172R}, the Tet-On 3G transactivator, and Puro^R to allow for simultaneous expression of the three genes. For the expression of POST-IT in zebrafish, QFGal4, Halo^{8KR}-PafA^{S126A,K172R}, 5×QUAS, EGFP, and SBP^{K4R}-sPup^{K61R}, were PCR amplified and assembled into the ClaI site of pUbi-QF-CK or pHsp-QF-CK, plasmids developed in our lab for use with the Tol2 transposon system. This assembly resulted in pUbi-QFGal4-2A-POST-IT and pHsp-QFGal4-2A-POST-IT, respectively. The P2A sequence was inserted between QFGal4 and Halo^{8KR}-PafA^{S126A,K172R}, as well as between EGFP and SBP^{K4R}-sPup^{K61R}.

Recombinant protein expression and purification

E. coli BL21(DE3) cells were transformed with a plasmid for expression of His-tagged or GST-tagged protein, and cultured in LB medium containing 100 µg/ml of either ampicillin or kanamycin overnight at 37 °C until the OD600 reached 0.6-0.7. Protein expression was induced by adding 0.1-0.5 mM IPTG and incubating at 16 °C for 16-20 h. The bacterial culture was then centrifuged at 6,000g for 15 min at 4 °C. The pellet was resuspended in *E. coli*-lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 5% glycerol, 1 mM PMSF), lysed by either sonication or a high-pressure homogenizer (AH-1500, ATS Engineering, China), and subsequently centrifuged at 24,000g at 4 °C for 30 min. PMSF stock solution (200 mM in isopropanol) was freshly added to the appropriate buffer to achieve the final concentration.

For Ni column purification via a 6×His-tag, the cleared supernatant was loaded onto Ni Sepharose 6 Fast Flow using a gravity flow. After extensive washing with more than 20 column volume (CV) of Ni-wash buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 0.1% Triton X-100, 10 mM imidazole, 1 mM PMSF), the recombinant protein was eluted with 2 CV of elution buffer 1-4 (50 mM Tris-HCl pH 8.0, 200 mM NaCl, varying concentrations of imidazole (250, 300, 350, 400 mM for elution buffer 1-4), 1 mM PMSF). Each fraction's purity was assessed by SDS-PAGE, followed by staining with R-250 Coomassie Brilliant Blue (CBB). The cleaner fractions were collected,

concentrated, and stored in storage buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT, 10% glycerol) through buffer exchange using Amicon Ultra Centrifugal Filter. All protein purification was performed via Ni column purification except for the following cases.

For GST purification via a GST-tag, the cleared supernatant was loaded onto glutathione resin and allowed to bind at 4 °C for 1 h. The resin was washed with more than 20 CV of Ni- wash buffer, with 10 CV of PreScission Protease reaction buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 mM DTT). 50 U of PreScission Protease (SLP00501, Smart- Lifesciences, China) was added and incubated at 4 °C overnight. The cleaved protein was collected, concentrated, and stored in storage buffer through buffer exchange using Amicon Ultra Centrifugal Filter.

For coVPS37C purification, a protocol using a mutant streptavidin, SAVSBPM18, was applied with minor modifications as previously shown (Wu et al., 2019 [\[14\]](#)). The synthesized SAVSBPM18 and DBD (dextran binding domain) were subcloned into the NcoI and XhoI sites of pET28a, creating pET-28a-SAVSBPM18-XTEN-DBD, which was then transformed into *E. coli* BL21(DE3) cells. The coVPS37C plasmid was transformed into the *E. coli* ArcticExpress (DE3) strain. The *E. coli* cells were cultured at 37 °C until the OD600 reached 0.6, at which point protein expression was induced by adding 0.2 mM IPTG. The *E. coli* cells were subsequently cultured at 12 °C overnight and processed to get cleared cell lysates as described above. *E. coli* cell lysates expressing SAVSBPM18-XTEN-DBD were prepared and mixed with coVPS37C lysates at a 1:4 ratio. After 1 h of incubation at 4 °C, the mixture was loaded onto Ni Sepharose 6 Fast Flow resin, washed with Ni-wash buffer, and eluted with elution buffer 2. The eluate was then mixed with Sephadex-G100 for 1 h at 4 °C, washed with Ni-wash buffer, and eluted with SBP elution buffer (10 mM biotin, 50 mM Tris-HCl pH 8.0, 300 mM NaCl). The final eluate was collected, concentrated, and stored in storage buffer through buffer exchange using Amicon Ultra Centrifugal Filter. All purified proteins were stored at -80 °C until use.

Cell culture and transfection

HEK293T (CRL-3216, ATCC, USA) and A375 (CRL-1619, ATCC, USA) cells were confirmed to be Mycoplasma-negative and cultured in Dulbecco's modified Eagle's medium (DMEM) (SH30243.01, Cytiva, USA), containing 4.5 g/L glucose, 4 mM glutamine, and 1 mM sodium pyruvate, supplemented with 10% FBS (SA211.02, Cellmax, China), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C with 5% CO₂. Transient transfections were performed using LipoJet (SL100468, SignaGen, USA), following the manufacturer's instruction. The identity of these cells was authenticated through SRT profiling.

Fluorescence polarization assay

The fluorescence polarization (FP) assay was conducted using a SpectraMax i3x microplate reader (Molecular Devices, USA) equipped with a fluorescence polarization cartridge (Ex. 485 nm, Em. 535 nm) and a grating (G) factor set to 1.5. Black opaque 96-well micro plates (Beyotime, China) were used for all experiments. For the in vitro time course measurement, 30 nM of Halo^{8KR}-PafA protein and 2 nM of HaloTag Alexa Fluor 488, Halo-AF488, were prepared in PBS buffer, and the parallel (I_v) and perpendicular (I_h) emission intensities were measured in kinetic mode at 20-s intervals for 30 min. The millipolarization units (mP) were calculated using the formula $mP = 1000 \times [(I_v - G \times I_h) / (I_v + G \times I_h)]$. In the competition assay with DH derivatives, 15 nM of Myc-Halo-PafA and 1 nM of Halo-AF488 were incubated with various DH derivatives in serial dilution at 37 °C for 30 min before FP measurement, with I_v and I_h measured at the endpoint. For the cellular binding assay, HEK293T cells were co-transfected with 4 µg of pTol2-myc-Halo-PafA-2ApA-CK2 per well in a 6-well plate and incubated for 48 h. Different DH derivatives at 1 µM or DMSO were added to each well and incubated for 3 h. Cell lysates were prepared in PBST (PBS with 0.5% Triton X-100), supplemented with a protease inhibitor cocktail (MB2678, MeilunBio, China), through brief

sonication followed by centrifugation. Protein concentration was determined by the BCA assay. Cleared cell lysates (5 µg/ul) were incubated with 2 nM Halo-AF488 in a 60 µl volume at 37 °C for 30 min. Iv and Ih were measured, and mP was calculated.

Western blot analysis

Cells transfected and/or treated with different compounds were harvested at the end of treatment. Cells were washed with ice-cold PBS and lysed with cell-lysis buffer that included RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5% glycerol), supplemented with 20 mM NaF, 2 mM Na₃VO₄, 0.4% SDS, 0.2% sodium deoxycholate (DOC), and a protease inhibitor cocktail. Following brief sonication, cell lysates were centrifuged at maximum speed for 10 min at 4 °C. The supernatant was collected, and the protein concentration was determined using the BCA assay. Samples were diluted to the same concentration, mixed with SDS sample buffer for SDS-PAGE, and boiled for 5-10 min. Proteins were separated by 6% or 8% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) and incubated overnight at 4 °C with the specific antibodies diluted in TBST containing 2% BSA: 1:5,000 anti-V5, 1:2,500 anti-HA, 1:5,000 anti-Myc, 1:5,000 anti-SRC, 1:10,000 anti-β-Actin, 1:5,000 Streptavidin-HRP. After three to four TBST washes, the membrane was incubated with a 1:10,000 HRP-conjugated secondary antibody against rabbit or mouse in TBST containing 5% skim milk for 1 h at room temperature. Following additional washes, the membranes were developed using chemiluminescence with Clarity Western ECL.

In vitro pupylation assay

For the self-pupylation assay, the reaction consisted of 1 µM Halo-PafA (or a mutant protein) and 10 µM of a Pup substrate in 20 µl of pupylation buffer (PBS supplemented with 15 mM MgCl₂ and 10 mM ATP), incubated at 37 °C for durations ranging from 10 to 180 min as specified in each figure, and stopped by the addition of 2× SDS sample buffer. Proteins were separated in three layers (4, 6, and 10%) Tricine-SDS-PAGE and stained with CBB. To test the effect of different DH derivatives on the pupylation of the target, 1 µM of a Halo-PafA derivative, 0.5 µM SRC(247-536)-2×V5, and 500 nM of a DH derivative were mixed in the pupylation buffer and incubated at room temperature for 30 min. Ten µM of a Pup substrate was then added and the mixture was incubated at 37 °C for another 30 min. The reaction was stopped by adding 2× SDS sample buffer, and the western blot assay was performed using an anti-V5 antibody. In the competition assay, a serial dilution of dasatinib from 0.2 µM to 10 µM was added along with 500 nM DH1 to the reaction before the addition of the Pup substrate. For the identification of pupylated lysine residues in PafA, 2.5 µM PafA and 20 µM HA-Pup were mixed in 50 µl of the pupylation buffer at 37 °C for 2 h. The reaction was stopped by adding 2× SDS sample buffer and separated by SDS-PAGE. CBB-stained bands were excised and analyzed by LC-MS/MS.

In cellulo pupylation assay

For the western blot assay, HEK293T cells were plated on 6-well plates and co-transfected as described below. For rapamycin-induced pupylation, HEK293T cells were co-transfected with 1 µg of pTol2-3×HA-FKBP-EGFP-pA, 1 µg of pTol2-3×V5-FRB-mKate2-PafA-2ApA-CK2, and 2 µg of pEF6a-HB-Pup (or one of pEF6a-SBP-sPup derivatives). After 24 h, 100 nM rapamycin was added to induce binding between HA-FKBP-EGFP and V5-FRB-mKate2-PafA. After 18 h, cells were harvested and lysed with cell-lysis buffer. Samples were then analyzed by western blot assay using antibodies against V5 and HA. To evaluate the levels of target labeling by different Halo-PafA variants or the effect of different drug-HTL derivatives, plates or dishes were coated with 10 µg/ml poly-D-lysine, and HEK293T cells were co-transfected with 1 µg of one of Halo-PafA variants, 2.5 µg of pEF6a-SBP^{K4R}-sPup^{K61R}, and 0.5 µg of pTol2-SRC(247-536)-2×V5. Twenty-four hours later, 250 (or 500 nM) of a DH derivative or 200 nM DC661-H1, unless stated otherwise, was added for an additional 24 h. Samples were then prepared for western blot analysis. For the competition assay with dasatinib,

100 nM DH1 and a serially increasing concentration of dasatinib, ranging from 0.025 to 25.6 μ M with a four-fold increment, were cotreated for 24 h. For the pulldown assay, cells were plated on 60 mm dishes, co-transfected with 2 μ g of one of Halo-PafA variants and 5 μ g of pEF6a-SBP^{K4R}-sPup^{K61R}, and lysed using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5% glycerol) supplemented with a protease inhibitor cocktail. To exogenously expression a target protein, 1 μ g of a target plasmid, such as SRC(247-536)-2 $\times$ V5 and VPS37C- 2 $\times$ V5, was included in the transfection. Cleared cell lysates were incubated with 50 μ l of streptavidin magnetic beads for 2 h at 4 $^{\circ}$ C. The beads were washed five times with wash buffer A (50 mM Tris-HCl pH 7.4, 400 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5% glycerol). Proteins were eluted with 2 $\times$ SDS sample buffer and processed for western blot analysis.

Preparation of proteomics samples for target-ID of DH5

HEK293T cells were plated on two 100 mm dishes for each condition and co-transfected next day with 8 μ g of pTol2-HA-Halo^{8KR}-PafA^{S126A,K172R} and 16 μ g of pEF6a-SBP^{K4R}-sPup^{K61R} per dish. After 24 h, treatments with DMSO, DH5 (250 nM), and a competition containing 250 nM DH5 and 2.5 μ M dasatinib were applied and incubated for an additional 24 h. Cells were harvested in 1 ml of RIPA buffer, supplemented with a protease inhibitor cocktail for each dish. Following brief sonication, cell debris was removed by centrifugation at maximum speed for 10 min at 4 $^{\circ}$ C. The supernatants were collected and mixed with 200 μ l of streptavidin magnetic beads that had been prewashed with RIPA buffer. The beads were washed five times with wash buffer A, then three times with wash buffer B (wash buffer A supplemented with 1 M urea), and eluted twice with 100 μ l of 5 mM biotin and 2.5 μ M dasatinib in wash buffer B for 1 h. The eluates were combined and precipitated using trichloroacetic acid (TCA)/DOC. The resultant protein pellets were neutralized with 1 M Tris-HCl pH 8.8, and separated by Tricine-SDS- PAGE, and the CBB-stained bands were excised, with those smaller than 10 kD being removed, and processed for mass analysis.

Preparation of proteomics samples for target-ID of DC661-H1 using SILAC

A stable HEK293T cells expressing the POST-IT system was generated using the PiggyBac transposon system. Briefly, HEK293T cells on a 60 mm dish were co-transfected with 8 μ g of PB-puro-CMV-POST-IT-Tet-SBP^{K4R}-sPup^{K61R}-iresGFP and 2 μ g of the Super PiggyBac Transposase expression vector (PB200PA-1, System Biosciences, USA). Four days later, cells were treated with 5 μ g/ml puromycin for about two weeks. Media containing puromycin were changed every 3–4 days.

The stable cell line was cultured in SILAC DMEM (280001200, Silantes, Germany) without arginine, lysine, and glutamine, supplemented with 10% dialyzed FBS (C3820-0100, VivaCell Biosciences, China) and 2 mM glutamine. The SILAC medium included 600 mg/L proline (P120032, Aladdin, China) to prevent the conversion of arginine to proline (Bendall et al., 2008 [\[1\]](#)).

The heavy medium, K8R10, contained 152.1 mg/L heavy labeled lysine K8 (¹³C₆ ¹⁵N₂) (211603902, Silantes, Germany) and 44.1 mg/L heavy labeled arginine R10 (¹³C₆ ¹⁵N₂) (201603902, Silantes, Germany). The light medium, K0R0, contained 146 mg/L normal lysine K0 (L113006, aladdin, China) and 42 mg/L normal arginine R0 (1119-34, aladdin, China). For SILAC experiments, cells were subcultured for at least 5 cell passages in either heavy or light DMEM medium.

The expression of POST-IT was induced by the addition of 100 ng/ml doxycycline. Two days later, 500 nM DC661-H1 was added to the heavy medium, or 500 nM DC661-H1 and 5 μ M DC660 to the light medium. After 24 h of incubation, cells were rinsed with ice-cold PBS and collected with 1 ml RIPA buffer, supplemented with a protease inhibitor cocktail, per dish. Cell debris was removed by centrifugation, and protein concentration was determined by the BCA assay. The protein concentration was adjusted to 2 mg/ml, and 200 μ l of prewashed streptavidin magnetic beads was added. After about 3 h of incubation at 4 $^{\circ}$ C, the beads were washed 5 times with wash buffer A, three times with wash buffer B, and eluted twice with 100 μ l of 5 mM biotin and 10 μ M DC660 in

wash buffer B for 1 h. The eluates were combined and precipitated using TCA/DOC. The resultant protein pellets were neutralized with 1 M Tris-HCl pH 8.8, and separated by Tricine-SDS-PAGE, and the CBB-stained bands were excised, with those smaller than 10 kD being removed, and processed for mass analysis.

LC-MS/MS analysis

Excised gels were sliced into small pieces of $\sim 1 \text{ mm}^3$, rinsed three times with Milli-Q water, decolorized with 50% acetonitrile and 100 mM NH_4HCO_3 , and dehydrated with 100% acetonitrile. The dehydrated gel pieces were then reduced with 10 mM DTT in 50 mM NH_4HCO_3 at 56 °C for 1 h, carboxymethylated with 55 mM iodoacetamide in the dark at room temperature for 30 min, and digested with trypsin at a 1:50 enzyme to protein mass ratio at 37 °C overnight. The peptides were collected, desalted with ZipTipC18 (ZTC18S096, Millipore, USA), dried under vacuum, and stored at -20 °C until MS analysis. The resulting tryptic peptides were analyzed by LC-MS/MS at the Institute of Hydrobiology (Chinese Academy of Sciences, Wuhan, China). LC-MS/MS data acquisition was performed using a Q Exactive HF-X mass spectrometer coupled with an Easy-nLC 1200 system (Thermo Fisher Scientific, USA). The peptide mixtures were initially loaded onto a C18 trap column and subsequently separated on a C18 reverse-phase analytical HPLC column, Acclaim PepMap C18 column (75 μm ID $\times$ 250 mm, 2 μm particle size, 100 Å pore size), Thermo Fisher Scientific, USA), employing a 100-min gradient program with mobile phase A (0.1% formic acid) and mobile phase B (80% acetonitrile, 0.1% formic acid). The gradient program was as follows: 0-65 min, 5-23% B; 65-85 min, 23-45% B; 85-86 min, 45-90% B; 87-90 min, 90% B; 90-90.1 min, 90-5% B; 90.1-100 min, 5% B, maintained at a constant flow rate of 300 nL/min. For data-dependent acquisition (DDA) mode analysis, each scan cycle consisted of one full-scan mass spectrum ($R = 60 \text{ K}$, $\text{AGC} = 3\text{e}6$, $\text{max IT} = 20 \text{ ms}$, $\text{scan range} = 350\text{-}1800 \text{ m/z}$) followed by 20 MS/MS events ($R = 15 \text{ K}$, $\text{AGC} = 2\text{e}5$, $\text{max IT} = 50 \text{ ms}$). The higher-energy collisional dissociation (HCD) collision energy was set to 28 for ion fragmentation. The isolation window for precursor selection was set to 1.6 Da. The former target ion exclusion was set for 25 s.

Mass spectrometry and data analysis

For the identification of DH5 targets, raw MS data were analyzed using MaxQuant (version 2.1.0.0, Max Planck Institute of Biochemistry) (Cox and Mann, 2008) with the Andromeda search engine. The MS data were aligned to the UniProt human protein database (Proteome ID: UP000005640). Additional sequences, including Halo^{8KR}-PafA^{S126A,K172R} and SBP^{K4R}-sPup^{K61R}, were integrated to identify potential proteomics contaminants. Variable modifications included oxidation (M), acetyl (Protein N-term), deamidation (NQ), and GGE (K). The false discovery rate (FDR) for both peptide and protein identification was set at 1%. The “Match Between run” option were enabled, with other settings at their default values. Missing values from identified proteins were filled through random imputation and further analyzed statistically to derive *P* values through unpaired SAM analysis using PANDA-view (Chang et al., 2018). Proteins commonly present under DMSO condition were considered nonspecific background interactions and excluded from further analysis.

For the SILAC experiments to identify DC661 targets, raw MS data were analyzed with FragPipe software v20.0 employing the MSFragger search engine (Kong et al., 2017). Alignment was performed against the UniProt human protein database (Proteome ID: UP000005640).

Halo^{8KR}-PafA^{S126A,K172R} and SBP^{K4R}-sPup^{K61R} sequences were incorporated as proteomics contaminants. The SILAC3 workflow was utilized with the default settings for light and heavy SILAC labels. Proteins detected fewer than 2 times across three biological replicates or exhibiting a high frequency of occurrence (> 0.15) in CRAPOME (Mellacheruvu et al., 2013) were filtered out from the analysis.

Microscale thermophoresis (MST) assay

SEPHS2-2×V5, VPS37C-2×V5, VPS29, TRAPPC3, TOM1, and TOM1L2 were labeled using Cy5-NHS ester (A100932, Sangon Biotech, China). Briefly, each target protein was diluted to 10 μM in PBS and underwent buffer exchange with PBS using a Zeba spin desalting column (89882, Thermo Scientific, USA) to remove Tris. The labeling reaction consisted of 80 μl of 10 μM target protein, 10 μl of 1 M NaHCO₃ pH 8.3, and 10 μl of 1 mM Cy5-NHS ester in DMSO, and was incubated at 4 °C overnight. The next day, unlabeled dyes were removed using Zeba spin desalting columns, and labeling efficiency was assessed by measuring the concentration of Cy5 with a UV-Vis spectrophotometer (SMA5000, Merinton, China). Dasatinib, DH5, HCQ, or DC660 was serially diluted in ligand buffer (PBS with 0.1% Tween-20). Cy5-labeled SEPHS2- 2×V5 (5 nM), VPS37C-2×V5 (5 nM), VPS29 (10 nM), TRAPPC3 (10 nM), TOM1 (10 nM), or TOM1L2 (10 nM) was mixed with the corresponding diluted compound in a total volume of 20 μl, and then loaded into Monolith premium capillaries (MO-K025, NanoTemper, Germany).

Binding affinity analysis was performed using a NanoTemper Monolith NT.115 instrument (NanoTemper, Germany). K_d values were obtained using MO.Affinity Analysis 2.3.0 software (NanoTemper, Germany).

Molecular docking simulation for protein-ligand interaction

The docking simulation was performed using CB-Dock2 (<https://cadd.labshare.cn/cb-dock2/index.php>), a web server that provides protein-ligand blind docking utilizing Autodock Vina (version 1.1.2). The blind docking simulation was conducted via protein-surface curvature- based cavity detection approach following the website procedures. The SEPHS2 structure information, ma-y60vo.cif, was obtained from the ModelArchive database (<https://doi.org/10.5452/ma-y60vo>) (Nunziata et al., 2019). The PDB files of VPS37 family proteins (Q8NEZ2, VPS37A; Q9H9H4, VPS37B; A5D8V6, VPS37C; Q86XT2, VPS37D) were downloaded from AlphaFold database (<https://alphafold.ebi.ac.uk/>), and the structure of the yeast ESCRT-I heterotetramer core (2P22) was retrieved from the RCSB protein data bank (<https://www.rcsb.org/>). Ligand files were downloaded from PubChem as sdf files. The superposition of VPS37C with other VPS37A/B/D proteins was performed using the US-align (Universal Structural alignment) website (<https://zhanggroup.org/US-align/>) (Zhang et al., 2022). UCSF ChimeraX 1.6.1 was employed for analyzing, editing, and visualizing the resulting PDB files.

Cellular thermal shift assay (CETSA)

HEK273T cells were cultured on a 100 mm dish and transfected with 10 μg of VPS37C-2×V5 plasmid. Forty-eight hours later, the cells were washed twice with ice-cold PBS, harvested in 1 ml of PBS buffer supplemented with 0.4% NP-40 and 0.5× protease inhibitor cocktail, incubated for 15 min with gently rotation at room temperature, and centrifuged at maximum speed for 15 min at 4 °C. The supernatant was transferred to a new 1.5 ml microtube, and protein concentration was determined by a BCA assay. Cell lysates were diluted to a concentration of 2 mg/ml and divided into two groups. The drug group was treated with 20 μM of DC660 or HCQ, while the control group received the same amount of DMSO. After a 20 min incubation at room temperature, cell lysates were gently mixed and aliquoted into PCR tubes with 40 μl each, then subjected to heat treatment for 4 min, ranging from 47 °C to 59 °C in 2 °C increments, and cooled to room temperature on a T100 thermal cycler (BioRad, USA). Subsequently, cell lysates were transferred to 1.5 ml microtubes and centrifuged at maximum speed for 20 min at 4 °C to precipitate unstable insoluble proteins. The supernatant was transferred to a new 1.5 ml microtube, and 10 μl of 5× SDS sample buffer was added to each tube. Samples were boiled at 95 °C for 5 min and processed for western blot analysis.

SRC kinase activity assay

Purified recombinant short SRC(247-536) (0.8 μ M), 10 μ M ATP, and 40 μ M of RR-SRC peptide, RRLIEDAEYAARG, (T510264-0001, Sangon Biotech, China) were prepared in SRC Kinase Buffer containing 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 0.1 mg/ml BSA, 2 mM $MnCl_2$, and 50 μ M DTT. After a 1-h incubation at room temperature, ATP levels were determined using the Kinase-Lumi luminescent kinase assay (S0150S, Beyotime, China) according to the manufacturer's instruction. To measure the IC_{50} values for dasatinib and DH5, dasatinib or DH5 in serial dilution was added to the SRC kinase reaction, and ATP levels were measured as outlined above. Relative SRC activities were calculated as compared to the DMSO condition, set as 100%.

Zebrafish husbandry

Wild-type AB zebrafish were maintained under standard conditions at 28.5 °C, with a 14-h light and 10-h dark cycle. Embryos were obtained from several matings, typically involving one male and one to two females, and were incubated in zebrafish E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) at 28.5 °C with the same light-dark cycle. The Animal Care and Use Committee of Wuhan University (No. AF078) provided approval for all procedures involving animals.

In vivo pupylation assay using zebrafish embryos

To express POST-IT in early zebrafish embryos, 20 pg of pUbi-QFGal4-2A-3 $\times$ Flag-POST-IT plasmid and 25 pg of transposase mRNA were injected into an embryo at the one-cell stage. The next day, GFP-positive and live embryos were selected, dechorionated with Pronase E (HY-114158, MedChemExpress, USA), and divided into two groups of approximately 1,000 embryos each. One group was treated with DMSO and the other with 10 μ M DH5 for 24 h. The embryos were then collected, rinsed with PBS, and lysed in cell-lysis buffer using a motorized plastic pestle. The resulting lysates were cleared by centrifugation, and protein concentrations were measured using the BCA assay. Samples were diluted to 2 mg/ml in 1 ml, mixed with 200 μ l of prewashed streptavidin magnetic beads, and incubate at 4 °C for 3 h. The beads were washed five times with wash buffer A. Proteins were eluted with 2 $\times$ SDS sample buffer and subsequently processed for western blot analysis.

Statistical analysis

Data plotting and statistical analysis were conducted using GraphPad Prism 9.5 software (GraphPad Software, Inc.). Data are presented as mean $\pm$ s.e.m. or mean $\pm$ s.d., as specified in each figure and its legend. *P* values were determined using Student's unpaired two-sided *t*-test, unless otherwise mentioned. *P* < 0.05 was considered as a statistical difference in this study.

Materials availability statement

Plasmids and cell lines generated in this study are available upon a reasonable request from the corresponding author (youngnam_jin@whu.edu.cn).

Data availability

All data generated or analyzed during this study are included in the paper and/or the supporting files. This study includes no data deposited in external repositories.

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Additional information

Author contributions

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Competing interest

The authors declare that no competing interests exist.

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Reviewer #2 (Public review):

Summary:

The study by Sun et al. introduces a useful system utilizing the proteasomal accessory factor A (PafA) and HaloTag for investigating drug-protein interactions in both in vitro (cell culture) and in vivo (zebrafish) settings. The authors presented the development and optimization of the system, as well as examples of its application and the identification of potential novel drug targets. However, the manuscript requires considerable improvements, particularly in writing and justification of experimental design. There are several inaccuracies in data description and a lack of statistics in some figures, undermining the conclusions drawn in the manuscript. Additionally, the authors introduced variants of the ligands and its cognate substrates, yet their use in different experiments appears random and lacks justification. It is challenging for readers to remember and track the specific properties of each variant, further complicating the interpretation of the results.

The conclusions of this paper are mostly backed by data, but certain aspects of data analysis and description require further clarification and expansion.

Comments on revisions:

We would like thank authors for submitting this revised version. We appreciate their inclusion of additional experiments, which convincingly demonstrate the absence of significant toxicity for in vivo applications. All our concerns and questions have been fully addressed. The clarity and quality of the writing have been substantially improved. We believe this innovative proximity labeling tool would be inspiring and valuable for the field.

<https://doi.org/10.7554/eLife.102667.2.sa2>

Reviewer #3 (Public review):

Summary:

This manuscript introduces POST-IT (Pup-On-target for Small molecule Target Identification Technology), a novel non-diffusive proximity tagging system for identifying target proteins in live cells and organisms. This technology preserves cellular context essential for capturing specific drug-protein interactions, including transient complexes and membrane-associated proteins. Using an engineered fusion of proteasomal accessory factor A (PafA) and HaloTag, POST-IT specifically labels proximal proteins upon binding to a small molecule, with extensive optimization to enhance specificity and efficiency.

Strengths:

The study successfully identifies known targets and discovers new binders, such as SEPHS2 for dasatinib and VPS37C for hydroxychloroquine, advancing our understanding of their mechanisms. Additionally, its application in live zebrafish embryos demonstrates POST-IT's potential for widespread use in biological research and drug development.

Comments on revisions:

The authors have addressed most of the issues I raised in my review. I have no further comments.

<https://doi.org/10.7554/eLife.102667.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

(1) The technology requires a halo-tagged derivation of the active compound, and the linked position will have a huge impact on the potential "target hits" of the molecules. Given the fact that most of the active molecules lack of structure-activity relationship information, it is very challenging to identify the optimal position of the halo tag linkage.

We appreciate your insightful comment. While finding the optimal position to attach a chemical linker to a small molecule of interest is indeed a challenging but necessary step, this is a common difficulty across all target-ID methods, except for those that are modification-

free, as we described in Discussion. However, modification-free approaches such as DARTS, CETSA, and TPP have their own limitations, such as low sensitivity and a high false-positive rate. Additionally, DARTS and SPROX are limited to use with cell lysates. Please refer to the introduction in our manuscript for more details on these approaches. On the other hand, synthesizing HTL derivatives is relatively straightforward compared to other modifications, and we provide helpful guidelines for chemical linker design, provided the optimal chemical moiety has been identified, which is crucial for target identification. We selected dasatinib and HCQ/CQ as model compounds because previous studies offered insights into their derivative synthesis. Our data also show that DH5 retains strong kinase inhibitory activity (Figure 4—figure supplement 2), and DC661-H1 demonstrates potent inhibition of autophagy (Figure 6—figure supplement 1). For novel compounds, conducting a thorough structure-activity relationship (SAR) study is essential to determine the optimal position for HTL derivative synthesis.

(2) Although POST-IT works in zebrafish embryos, there is still a long way to go for the broad application of the technology in other animal models.

Thank you for your constructive comment. Yes, there is still a long way to go in developing the POST-IT system for broader applications in other animal models, especially in mice. However, we hope that our study provides valuable insights and inspiration to scientists and experts for applying the POST-IT system in various models. We are also committed to further improving its applicability.

(3) The authors identified SEPHS2 as a new potential target of dasatinib and further validated the direct binding of dasatinib with this protein. However, considering the super strong activity of dasatinib against c-Src (sub nanomolar IC50 value), it is hard to conclude the contribution of SEPHS2 binding (micromolar potency) to its antitumor activity.

Thank you for your insightful comment. We agree that the anticancer activity of dasatinib primarily results from inhibiting tyrosine kinases such as SRC and ABL. However, SEPHS2 contains an “opal” termination codon, UGA, at the 60th amino acid residue, which codes for selenocysteine. Due to the technical challenge of expressing selenoproteins in *E. coli*, we mutated it to cysteine for expression in *E. coli* to avoid premature translation termination, as described in the Materials and Methods section. Although the purified recombinant SEPHS2 shows a Kd of about 10 μ M for dasatinib, the binding affinity to endogenous SEPHS2 may be higher since selenocysteine is larger and more electronegative than cysteine. This presents an interesting area for future investigation. Furthermore, our study of dasatinib’s binding to SEPHS2 could help facilitate the development of new SEPHS2 inhibitors, potentially targeting the active site of SEPHS2.

Reviewer #3 (Public review):

(1) Target Specificity: It is crucial for the authors to differentiate between the primary targets of the POST-IT system and those identified as side effects. This distinction is essential for assessing the specificity and utility of the technology.

Thank you for your insightful comment. Drugs inevitably bind to various proteins with differing affinities, which can contribute to both side effects and beneficial outcomes. Typically, the primary targets exhibit high affinities. In this manuscript, we ranked the identified protein targets of DH5 based on affinity from mass spectrometry and p-values (Fig. 5A), and for DC661-H1, we used the SILAC ratio (Fig. 6A). We also individually assessed many drug-protein binding affinities using the MST assay, as well as in vitro and in cellulo assays, demonstrating their specificity. Moreover, we believe it is essential to identify as many

protein targets as possible at physiological drug concentrations to better understand the drug's side effects. Of course, further investigation is required to assess the roles and effects of these target proteins.

(2) In Vivo Target Identification: The manuscript lacks detailed clarity on which specific targets were successfully identified in the in vivo experiments. Expanding on this information would provide a clearer view of the system's effectiveness and scope in complex biological settings.

Thank you for your insightful comment regarding in vivo target identification. In this manuscript, we utilized a cell line as the primary method for in vivo target identification and validation after optimizing our system in test tubes. We successfully validated many of the targets identified using our POST-IT system (Figure 6—figure supplement 3). To demonstrate the proof of principle for in vivo application, we employed zebrafish embryos as an in vivo model, showing that endogenous SRC can be effectively pulled down by DH5 treatment (Fig. 7). While we could have explored the entire proteome to identify endogenous target proteins in zebrafish that bind to DH5 or dasatinib, we felt this would extend beyond our original scope, given that we have already demonstrated POST-IT's ability to identify target proteins for dasatinib. Specific target identification and validation are crucial when using zebrafish for drug discovery. Additionally, we acknowledge that drugs likely interact with a range of protein targets in living organisms and may undergo metabolism and interactions within the circulatory system, which we address in our discussion.

(3) Reproducibility and Scalability: Discussion on the reproducibility of the POST-IT system across various experimental setups and biological models, as well as its scalability for larger-scale drug discovery programs, would be beneficial.

Thank you for the suggestion. While our system has shown high reproducibility in our experiments, further improving both reproducibility and scalability would be advantageous. One potential approach to address this is through the generation of stable-expressing cell lines and transgenic zebrafish lines, which we have discussed in the revised manuscript. Establishing stable cell lines with robust POST-IT expression could enhance scalability for drug discovery applications.

(4) Quantitative Analysis: A more detailed quantitative analysis of the protein interactions identified by POST-IT, including statistical significance and comparative data against other technologies, would enhance the manuscript.

Thank you for your suggestion. In our assessment of drug-protein affinity, we included Kd values as quantitative measures using MST assays. The protein targets of dasatinib identified through mass spectrometry are also accompanied by p-values for quantitative analysis (Fig. 5A), and the detailed procedures are described in the Material and methods section. While it is challenging to provide direct comparative data against other technologies, our system successfully identified many known target proteins for dasatinib, as well as SEPHS2 and VPS37C as new targets for dasatinib and for HCQ/CQ, respectively, which were not detected by other methods.

(5) Technological Limitations: The authors should discuss any limitations or potential pitfalls of the POST-IT system, which would be crucial for future users and for guiding subsequent improvements.

Thank you for your insightful suggestion. We agree that clearly defining the technological limitations is important. Therefore, we have expanded our original discussion on the limitations of our POST-IT system (Discussion section, paragraph 6).

(6) Long-Term Stability and Activity: Information on the long-term stability and activity of the POST-IT components in different biological environments would ensure the reliability of the system in prolonged experiments.

Yes, this is an important question. We did not notice any stability or toxicity issues with Halo-PafA and Pup substrates in HEK293T cells or zebrafish, which is an important factor for stable cell lines and transgenic zebrafish lines. However, HTL derivatives of the drug could be toxic or unstable due to the nature of the drug or its metabolism, which needs to be taken into account when designing experiments, and we have included this in the Discussion.

(7) Comparison with Existing Technologies: A detailed comparison with existing proximity tagging and target identification technologies would help position POST-IT within the current landscape, highlighting its unique advantages and potential drawbacks.

We appreciate your valuable feedback and agree that such comparisons are crucial. We have included a detailed overview and comparison of existing proximity-tagging systems and their related target identification technologies in the Introduction (lines 78-100) and Discussion (lines 391-412), highlighting their respective pros and cons. Additionally, we have expanded the discussion to further compare these technologies with our POST-IT system, addressing its advantages and limitations (lines 378-390, lines 448-467). We hope this provides sufficient context and information to effectively position POST-IT among the landscape of proximity-tagging target identification technologies.

(8) Concerns Regarding Overexposed Bands: Several figures in the manuscript, specifically Figure 3A, 3B, 3C, 3F, 3G, Figure 4D, and the second panels in Figure 7C as well as some figures in the supplementary file, exhibit overexposed bands.

We appreciate your astute observation regarding the overexposed bands and apologize for any confusion. The “overexposed” bands represent the unpupylated proteins, while the bands above them correspond to the pupylated proteins. We intended to clearly show both pupylated and unpupylated bands, although the latter are generally much weaker. We are currently working on further improving our POST-IT system to enhance pupylation efficiency.

(9) Innovation Concern: There is a previous paper describing a similar approach: Liu Q, Zheng J, Sun W, Huo Y, Zhang L, Hao P, Wang H, Zhuang M. A proximity-tagging system to identify membrane protein-protein interactions. Nat Methods. 2018 Sep;15(9):715-722. doi: 10.1038/s41592-018-0100-5. Epub 2018 Aug 13. PMID: 30104635. It is crucial to explicitly address the novel aspects of POST-IT in contrast to this earlier work.

Thank you for bringing this to our attention. Proximity-tagging systems like BioID, TurboID, NEDDylator, and PafA (Liu Q et al., Nat Methods 2018) were initially developed to study protein-protein interactions or identify protein interactomes, as these applications are of broader interest and generally easier to implement. However, applying proximity-tagging systems for small molecule target identification requires significant optimization. As described in the introduction (lines 78-100), target protein identification systems have since been developed using TurboID and NEDDylator (Tao AJ et al., Nat Commun 2023; Hill ZB et al., J Am Chem Soc 2016). It is conceivable that a PafA-based proximity-tagging system could also be adapted for target-ID, and other groups may pursue this approach in the future. Although the PafA-Pup system shows great promise for target-ID applications, extensive optimization was needed to enable its use for this purpose. Finally, we demonstrate that POST-IT offers distinct advantages over other proximity-tagging-based target-ID systems. For more details, please refer to the introduction and discussion sections.

Recommendations for the authors:

Reviewer #2 (Recommendations for the authors):

(1) Figure 1- Figure Supplement 1A: The Pup substrate "HB-Pup" is mentioned, but the main text or figure legend provides no introduction or description.

We appreciate your astute observation. We have added a description in the main text and figure legend as follows: "...and used HB-Pup as a control, which contains 6His and BCCP at the N terminus of Pup" in the main text (line 142) and "HB, TS, and SBP refer to 6His and BCCP, twin-STII (Strep-tag II), and streptavidin binding peptide, respectively." in the Figure 1-figure supplement 1A.

(2) Figure 1 - Figure Supplement 3B: The authors used TS-sPupK61R as a substrate but did not explain why. The main text mentions that mutating sPup alone did not affect polypupylation, raising the question of why TS-sPupK61R was used in this figure. Furthermore, while the authors state that polypupylation becomes evident after 1 hour of incubation (more pronounced after 2 or 3 hours), the reactions here were conducted for only 30 minutes.

Thank you for your question. Figure 1 - Figure Supplement 3B was conducted to test self-pupylation levels in the different Halo-PafA derivatives. For this purpose, we could use any Pup substrate such as SBP-sPup and SBPK4R-sPupK61R, instead of Ts-sPup and TS-sPupK61R, as they do not show any differences in pupylation activity. We chose Ts-sPup and TS-sPupK61R simply because any Pup substrates could be used for this purpose. Similarly, we did not need to incubate the reaction for a longer time to detect polypupylation, as our intention was to test "self-pupylation". We demonstrated in Figure 1 – figure supplement 2 that polypupylation is dependent on the number or position of lysine residues in Pup substrate or tags. The results clearly showed that self-pupylation was almost completely abolished by the Halo8KR mutation. To clarify this, we added the following description in lines 168-169: "Ts-sPup and TS-sPupK61R were chosen as sPup substrates for this experiment, although any Pup substrates could have been used. The levels of self-pupylation were assessed."

(3) Line 156: The statement that "the TS-tag completely abolished polypupylation in TS-sPup" is inaccurate. Using TSK8R-sPupK61R as the substrate, several bands appear, which likely represent Halo-PafA with varying degrees of polypupylation. Some bands also appear to correspond to those seen when using TS-sPup as a substrate. The authors should clarify how they distinguish between multipupylation and polypupylation in this case.

We sincerely appreciate your insight into clarifying the distinction between multipupylation and polypupylation. Polypupylation refers to the addition of a new Pup onto a previously linked Pup on the target protein, akin to polyubiquitination. In contrast, multipupylation involves multiple single pupylations at different positions on the target proteins. Since pupylation occurs exclusively at lysine residues in tag-Pup substrates, mutating all lysine residues to arginine, as in TSK48R-sPupK61R, prevents the mutant tag-Pup from linking to another Pup. This means that only single pupylation can proceed with this type of mutant Pup substrate. If multiple pupylated bands are observed with this mutant substrate, it indicates "multipupylation" rather than "polypupylation", as shown in Figure 1-figure supplement 2D. The same applies to the pupylation bands in Figure 1-figure supplement 2E and F, as sSBP-sPupK61R and SBPK4R-sPupK61R lack lysine residues. By comparing these multipupylation bands, it is also possible to distinguish them from polypupylation bands,

which are marked by yellow arrows. However, after 2-3 pupylation bands, higher-order bands become increasingly difficult to distinguish.

To clarify the mutation in the TS-tag, we revised the sentence in line 156 from “However, further mutations within the TS-tag completely abolished polypupylation in TS-sPup” to “However, further mutations of two lysine residues within the TS-tag, creating TSK8R-sPupK61R, completely abolished polypupylation in TS-sPup”. Additionally, we have inserted sentences in line 152 to define polypupylation and multipupylation, as described here.

(4) Line 160: Similar to the above concern about line 156, the claim that SBPK4R and sSBP completely prevented polypupylation is unconvincing and requires more supporting evidence.

Thank you for raising this concern. As mentioned above, both SBPK4R and sSBP lack lysine residues required for pupylation. As a result, these mutants can only undergo multiple single pupylations on the lysine residues of the target protein, which leads to “multipupylation”. In Figure 1-figure supplement 2E and F, pupylation bands by sSBP-sPupK61R or SBPK4R-sPupK61R do not display doublet bands (one from multipupylation and the other from polypupylation), as seen with SBP-sPup, marked by yellow arrows. Notably, Halo-PafA containing polypupylated branches migrates more slowly than one with an equal number of multipupylation events. To clarify this point, we have added the phrase “as shown in sSBP-sPupK61R and SBP4KR-sPupK61R” at the end of the sentence in line 160.

(5) Lines 176-177: The authors claim that PafAS126A exhibited reduced polypupylation compared to PafA, but given that PafAS126A may reduce depupylase activity, how could it reduce polypupylation levels? Moreover, it is hard to find any data supporting this conclusion in Figure 1 - Figure Supplement 3B.

We appreciate your insightful comment. At this point, we do not fully understand how the mutation that reduces depupylase activity also decreases polypupylation. It is possible that PafAS126A has a lower preference for pupylated Pup as a prey, which is required for polypupylation, since depupylase activity depends on recognizing pupylated Pup as a prey to remove it. Nonetheless, Halo-PafAS126A shows reduced levels of higher molecular weight bands compared to Halo-PafA, as shown in Figure 1-figure supplement 3B, while exhibiting increased pupylation in lower molecular weight bands, which represent either multipupylation or low-degree polypupylation. Since higher molecular weight bands (> 150 kD) are likely due to polypupylation, this result suggests reduced polypupylation and increased multipupylation in Halo-PafAS126A. To clarify this in the main text, we have added the following description in line 177: “as evidenced by the decreased levels of high molecular weight bands and an increase in low molecular weight bands”

(6) POST-IT system in cellulo validation: The system was developed using the Halo-tag, yet the in-cell validation uses FRB and FKBP instead, without explaining this switch. This inconsistency makes the logic of the experiment unclear.

We appreciate your insightful comment. The interaction between rapamycin and FRB or FKBP is known to be highly specific and robust, making this system useful in various biological contexts. Due to this property, rapamycin can induce interaction between two proteins when one is fused with FRB and the other with FKBP. Before testing or optimizing the POST-IT system in cells, we hypothesized that using the rapamycin-induced interaction between FRB and FKBP could introduce pupylation of the target protein, provided that PafA is fused with FRB or FKBP and the target protein is fused with the other. The results demonstrate that PafA can introduce pupylation of the target protein in a proximity-dependent manner via this chemically induced interaction. To further clarify this in the main

text, we modified the original sentence in lines 214-216 as follows: “To mimic drug-target interaction-induced pupylation in live cells and assess the potential of PafA as a proximity-tagging system for target-ID, we incorporated the rapamycin-induced interaction between FRB and FKBP into our PL system, as this interaction between a small molecule and a protein is known to be highly specific and robust (Figure 3—figure supplement 1A).”

(7) Line 209: The authors decided to use the SBP-tag for further studies due to better performance, but in Figure 3 - Figure supplement 1, they still used the unintroducted HB-Pup as the substrate, which is confusing and lacks explanation.

Thank you for raising your question. The SBP-tag is not superior to the TS-tag in terms of pupylation activity. However, the TSK8R mutant cannot bind to Strep-Tactin beads, while the SBP mutants, SBPK4R and sSBP, can bind to streptavidin. Therefore, we chose the SBP-tag instead of the TS-tag for further studies as a Pup substrate in POST-IT system, as we needed to pull down the target proteins. HB-Pup is consistently used as a control throughout various experiments, as it is the original Pup substrate. In Figure 3-figure supplement 1B and C, HB-Pup was used to test chemically induced pupylation by PafA. In these cases, it was not so critical which Pup substrate was chosen. Furthermore, we compared HB-Pup and different SBP-sPup substrates in Figure 3-figure supplement 1D, where HB-Pup was used as a control or for comparison. Although pupylation bands with HB-Pup appear more robust, this substrate contains multiple lysine residues, leading to high levels of polypupylation. To make it clear, we modified the sentence in line 209 to “Therefore, we decided to use the SBP-tag as a Pup substrate in the POST-IT system for further studies.”

(8) Line 220: Both SBP-sPup and SBPK4R-sPupK61R are described as exhibiting efficient pupylation, but the data show mostly self-pupylation and little to no pupylation of the target protein.

Thank you for your concern. However, pupylation of the target protein is actually quite substantial, as the intensities of the free form and pupylated proteins are relatively similar, as shown in the upper panel of Figure 3-figure supplement 1D. Self-pupylation is always much higher than target pupylation, because PafA constantly pupylates itself, whereas pupylation of the target protein occurs only through interaction. Furthermore, V5-FRB-mKate2-PafA contains many lysine residues, which increases the levels of self-pupylation.

(9) Lines 222-224: The authors chose SBPK4R-sPupK61R to avoid polypupylation, although SBP-sPup did not cause detectable polypupylation. Neither substrate caused pupylation of the target protein, so the rationale behind this choice is unclear.

Thank you for raising your question. Similar to the above comment (#8), please refer to the pupylation bands of the target protein, as shown in the upper panel of Figure 3-figure supplement 1D. The pupylation band of the target protein is quite remarkable, as the intensities of the free form and pupylated proteins are comparable. Additionally, there are no multiple pupylation bands in either case, except for one additional weak multipupylation band, indicating no polypupylation by SBP-sPup, which does not have K-to-R mutations. Of course, SBPK4R-sPupK61R can only undergo single pupylation, as it does not contain lysine residues. Although we did not observe polypupylation by SBP-sPup in this experimental condition, it is possible that SBP-sPup may cause polypupylation under different experimental conditions or with other target proteins. Since SBPK4R-sPupK61R exhibits comparable pupylation of the target protein at least in this experiment setting as SBP-sPup, we selected SBPK4R-sPupK61R as the Pup substrate for POST-IT system to avoid any potential polypupylation that could be caused by SBP-sPup in other cases. We believe that polypupylation can introduce bias into the analysis and hinder the comprehensive discovery of additional target proteins for small molecules.

(10) Line 224: The authors conclude that rapamycin greatly reduced self-pupylation, but the supporting data are unclear.

Thank you for your constructive comments on our manuscript. Please refer to the lower panel of Figure 3-figure supplement 1D. When using either SBPK4R-sPupK61R or SBP-sPup, rapamycin treatment results in reduced levels of self-pupylation compared to the no-treatment control. However, we did not observe this reduction with HB-Pup and do not know the reason. To clarify this in the main text, we added the following description to the end of the sentence: “when using either SBPK4R-sPupK61R or SBP-sPup, as shown in the lower panel of Figure 3—figure supplement 1D”

(11) Line 234: The authors selected an 18-amino acid linker, but given that linkers longer than 10 amino acids enhance labeling, this choice should be explained.

Thank you for raising your question. In fact, a linker of 10 amino acids (aa) or longer is likely to behave similarly. We chose an 18 aa linker instead of a 40 aa linker primarily for the convenience of cloning and to reduce the potential for DNA sequence recombination associated with longer repeats. Additionally, a longer, flexible linker may behave like an intrinsically disordered protein (Harmon et al., 2017), which can lead to unwanted protein-protein interactions or phase separation. To elaborate on this, we added the following sentences after the sentence in line 233-235: “We chose the 18-amino acid linker instead of the 40-amino acid linker for easier cloning and to lower the risk of DNA recombination from longer repeats. Additionally, a longer, flexible linker may behave like an intrinsically disordered protein (Harmon et al., 2017), an unwanted feature for target-ID.”

(12) S126A and K172R mutations: The authors claim that these mutations additively enhanced pupylation under cellular conditions, but in Figure 3B, the band intensities appear similar for the wild-type and mutant versions.

Thank you for raising your concern. Although a single pupylation band appears similar among the three different Halo-PafA proteins, multipupylation bands are slightly but noticeably increased by the S126A and K172R mutations compared to Halo8KR-PafA. Since we used SBPK4R-sPupK61R as a Pup substrate, all higher molecular weight bands result from multipupylation rather than polypupylation. This illustrates why it is preferable to use SBPK4R-sPupK61R over SBP-sPup, as the pupylation bands with SBP-sPup are mixtures of poly- and multipupylation, making it difficult to assess levels of target labeling. To clarify this in the main text, we added the following description after the sentence in line 236: “as the higher molecular weight multipupylation bands are slightly but noticeably increased with these mutations compared to Halo8KR-PafA”

(13) Line 263: The authors selected DH5 for further experiments due to its efficiency, but the data suggest that the performance of DH1 to DH5 is similar.

We appreciate your question about the different dasatinib HTL derivatives. However, our data clearly show that DH2-5 derivatives bind significantly more effectively to Halo-PafA in vitro and in live cells compared to DH1 (Figure 4A and B). Additionally, the DH2-5 derivatives result in dramatically increased pupylation of the target protein in vitro and noticeable enhancement in live cells (Figure 4C and D). Among DH2 to DH5, there is no obvious difference in binding to Halo-PafA or pupylation of the target protein. Therefore, we chose DH5, as we believe that the longer linker in DH5 may facilitate the binding of a more diverse range of target proteins to dasatinib, enabling the discovery of additional target proteins.

(14) Line 309: The authors introduce HCQ and CQ as important drugs but then investigate the mechanism using DC661 without introducing or justifying the choice of this compound.

Thank you for your point. We explained the reason to choose DC661, a dimer form of CQ, instead of CQ for the synthesis of an HTL derivative in line 310. “assuming that a dimer would enhance binding affinity as previously described.” As the dimer forms of a drug or a small molecule such as testosterone dimers, estrogen dimers, and numerous anticancer drug dimers have been often developed to enhance drug effects (Paquin A et., *Molecules* 2021). Similarly, dimer forms of HCQ/CQ have been introduced and shown to be more potent (Hrycyna CA et al., *ACS Chem Biol* 2014; Rebecca VW et al., *Cancer Discovery* 2019). We expected that using a dimer form might offer higher probability to identify target proteins for HCQ/CQ.

(15) The authors suggest that multipupylation levels were enhanced but do not explain whether this might benefit the system or introduce other issues. Clarifying this point would provide valuable insight for potential users of this system.

Thank you for your thoughtful suggestion. Polypupylation likely leads to biased enrichment of a limited set of target proteins, and its levels may not correlate with the binding affinity of target proteins to the small molecule of interest, features that can negatively impact target-ID. In contrast, multipupylation may be correlated with binding affinity or interaction frequency, as we observed increased levels of multipupylation with higher Pup concentrations and longer incubation times. This suggests that target proteins with multiple lysines in proximity to PafA can be sequentially pupylated, starting with the most accessible lysine. However, if a target protein has only one accessible lysine, pupylation will occur only once, regardless of the protein's affinity to the small molecule. In summary, while polypupylation may be a drawback for target-ID, multipupylation could be useful for both target-ID and understanding binding mode. To elaborate on this, we added the following additional explanation after the sentence in line 152: “, whereas multipupylation is more likely correlated with binding affinity or interaction frequency.”

(16) The author should address whether the Halotag ligand modification of the drug alters the binding properties between the drug and targets. That may be causing artifact binding of the drug and other proteins.

Thank you for your insightful comment. Yes, it is true that chemical modifications of the small molecule of interest, such as linker derivatization (e.g., HTL) or photo-affinity labeling, generally lead to reduced activity or affinity compared to the original molecule. Synthesizing a derivative is a common challenge across all target-ID methods, except for modification-free approaches, as we mentioned in the Discussion. However, modification-free methods like DARTS, CETSA, and TPP have their own limitations, including low sensitivity or high false positive rates. Identifying the optimal position for chemical modification on the small molecule of interest is critical. We chose dasatinib and HCQ/CQ as model compounds, because previous studies provided insights into their derivative synthesis. In addition, our data show that DH5 retains robust kinase inhibitory activity (Figure 4-figure supplement 2), and DC661-H1 exhibits potent autophagy inhibition (Figure 6-figure supplement 1). For novel compounds, a thorough structure-activity relationship study is essential to identify the optimal position for HTL derivative synthesis.

(17) The author stated there is no observable toxicity in zebrafish without providing a detailed analysis or enough data. Further analysis of the expression of Halo-PafA and its

substrate sPup influence on toxicity or side effects to the living cells or animals would be needed. It is important for in vivo applications.

Thank you for your constructive suggestion. We have now included additional experimental data in Figure 7-figure supplement 1, showing no toxicity in zebrafish embryos expressing the POST-IT system. We assessed toxicity in two ways: by injecting the POST-IT DNA plasmid into one-cell-stage embryos for acute expression, and by using embryos from transgenic zebrafish expressing POST-IT under a heat-shock inducible promoter. Neither the injection nor the heat-shock activation of POST-IT expression resulted in any noticeable toxicity.

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