Cell-surface tethered promiscuous biotinylators enable comparative small-scale surface proteomic analysis of human extracellular vesicles and cells

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Abstract Characterization of cell surface proteome differences between cancer and healthy cells is a valuable approach for the identification of novel diagnostic and therapeutic targets. However, selective sampling of surface proteins for proteomics requires large samples (>10e6 cells) and long labeling times. These limitations preclude analysis of material-limited biological samples or the capture of rapid surface proteomic changes. Here, we present two labeling approaches to tether exogenous peroxidases (APEX2 and HRP) directly to cells, enabling rapid, small-scale cell surface biotinylation without the need to engineer cells. We used a novel lipidated DNA-tethered APEX2 (DNA-APEX2), which upon addition to cells promoted cell agnostic membrane-proximal labeling. Alternatively, we employed horseradish peroxidase (HRP) fused to the glycan binding domain of wheat germ agglutinin (WGA-HRP). This approach yielded a rapid and commercially inexpensive means to directly label cells containing common N-Acetylglucosamine (GlcNAc) and sialic acid glycans on their surface. The facile WGA-HRP method permitted high surface coverage of cellular samples and enabled the first comparative surface proteome characterization of cells and cell-derived small extracellular vesicles (EV), leading to the robust quantification of 953 cell and EV surface annotated proteins. We identified a newly-recognized subset of EV-enriched markers, as well as proteins that are uniquely upregulated on Myc oncogene-transformed prostate cancer EVs. These two cell-tethered enzyme surface biotinylation approaches are highly advantageous for rapidly and directly labeling surface proteins across a range of material-limited sample types.

Introduction

The cell surface proteome, termed the surfaceome, serves as the main communication hub between a cell and the extracellular environment (Wollscheid *et al.*, 2009). As such, this cellular compartment often reveals the first signs of cellular distress and disease, and is of substantial interest to the medical community for diagnostic and therapeutic development (Leth-Larsen *et al.*, 2010).

Table 1. Current methods available for cell surface biotinylation.

Method	Protocol Length (time)	Selectivity	Sample Size Requirement
Biocytin Hydrazide	+++	+++	+++
Sulfo-NHS-LC-LC-biotin	++	+	+
APEX2/HRP	+	+	+

38 The precise and comprehensive profiling of the surfaceome, termed surfaceomics, provides critical
 39 insights for our overall understanding of human health and can inform drug development efforts.
 40 Several strategies have emerged for either selective or comprehensive surfaceomics, including bio-
 41 cytin hydrazide labeling of surface glycoproteins (*Wollscheid et al., 2009*), chemical biotinylation of
 42 lysines via NHS-ester labeling (*Huang, 2012*), and promiscuous biotinylation fusion proteins (APEX2,
 43 BioID, SPPLAT) (*Rees et al., 2015a; Sears et al., 2019; Wollscheid et al., 2009*). Membrane protein
 44 enrichment is a necessary step in surfaceomics, due to the inherent low abundance of membrane
 45 proteins compared to cytosolic proteins, and their identification can be overwhelmed by cytosolic
 46 contaminants. While each of these strategies robustly label surface proteins, they: (1) require large
 47 sample inputs (biocytin hydrazide), (2) require production of genetically engineered cells (APEX2,
 48 BioID), (3) label only partner proteins by binding targeting antibodies fused to APEX2 or HRP (SP-
 49 PLAT), (4) require extensive sample manipulation (biocytin hydrazide), or (4) exhibit increased non-
 50 specific labeling (NHS-ester, Table 1) (*Bausch-Fluck et al., 2012; Elschenbroich et al., 2010; Griffin
 51 and Schnitzer, 2011; Kuhlmann et al., 2018; Li et al., 2020b*). Moreover, many of these methods
 52 are not able to capture short and transient changes that occur at the cell surface, such as bind-
 53 ing, adhesion, assembly, and signaling (*Kalxdorf et al., 2017*). These current methods complicate
 54 the direct characterization of small clinical samples such as extracellular vesicles in patient serum.
 55 As biological research increasingly depends on animal models and patient-derived samples, the re-
 56 quirement for simple and robust methods amenable to direct labelling of material-limited samples
 57 for proteomic analysis will become paramount.

58 Exosomes and other small extracellular vesicles (EVs) are produced by both healthy and dis-
 59 eased cells (*Colombo et al., 2014*). In cancer, these small EVs contribute to tumor growth and
 60 metastasis, modulate the immune response, and mediate treatment resistance (*Al-Nedawi et al.,
 61 2008; Edgar, 2016; Kalluri and LeBleu, 2020; Shurtleff et al., 2018*). Consequently, these extracel-
 62 lular vesicles are a focus of intense clinical investigation. Recent studies suggest that small EVs
 63 incorporate proteins and RNA from the parent tumor from which they originate (*Lin et al., 2015;
 64 Soung et al., 2017*), and certain proteins may be preferentially shuttled into EVs (*Poggio et al., 2019*).
 65 There is also strong evidence that cancer-derived EVs are unique from the EVs derived from healthy
 66 surrounding tissues, and therefore represent a promising target for non-invasive, early-detection
 67 diagnostics or EV-focused therapies (*Kalluri and LeBleu, 2020; Skog et al., 2008; Zhou et al., 2020*).
 68 However, strategies for the unbiased profiling of small EV membrane proteomes remain limited.
 69 Isolation of high-quality, enriched small EV populations is challenging, requiring numerous centrifu-
 70 gation steps and a final sucrose gradient isolation, precluding the use of current labeling methods
 71 for membrane proteome characterization (*Poggio et al., 2019; Shurtleff et al., 2018*). Strategies
 72 to characterize the surface proteome of small EVs would propel biomarker discovery and enable
 73 the differential characterization of small EVs from that of the parent cell. These important studies
 74 could help illuminate mechanisms underlying preferential protein shuttling to different extracellu-
 75 lar vesicle populations.

76 Here, we functionalize the promiscuous biotinylation, APEX2 and HRP, as non-cellularly en-
 77 coded exogenous membrane tethering reagents for small-scale surfaceomics, requiring <5e5 cells.
 78 This method is 10-100 fold more rapid than other existing protocols and requires fewer wash steps
 79 with less sample loss. Likewise, due to its selectivity towards tyrosines, it is not hindered by vari-
 80 ability in individual protein glycosylation status (*Leth-Larsen et al., 2010*) or by impeding complete

81 tryptic peptide cleavage through modification of lysines (*Hacker et al., 2017*), like biocytin hydrazide
82 or NHS-biotin methods, respectively. Using this robust new strategy, we performed surfaceomics
83 on cells and corresponding small EVs from a cellular model of prostate cancer using the prostate
84 epithelial cell line, RWPE-1 with or without oncogenic Myc induction. While certain proteins show
85 increased expression in both parental cell and EV surfaces, a subset of proteins were found to be ei-
86 ther pan-EV markers (ITIH4, MFGE8, TF, DSG1, TSPAN14, AZGP1, and IGSF8) or selectively enriched
87 with Myc overexpression in cancer-derived EVs (ANPEP, SLC38A5, FN1, SFRP1, CDH13, THBS1, and
88 CD44). These differentially-regulated proteins pose interesting questions related to preferential
89 protein shuttling, and the proteins upregulated in both cellular and EV contexts reveal candidates
90 for early-stage urine or serum-based detection without invasive surgical intervention. We believe
91 these simple, rapid, and direct surfaceomic labeling tools may be broadly applied to small-scale
92 surfaceomics on primary tissues.

93 Results

94 Generation of promiscuous cell-surface tethered peroxidases for exogenous addi- 95 tion to cells

96 Both APEX2 and HRP are broadly used promiscuous proximity biotinylation reagents that label nearby ty-
97 rosine residues in proteins through a radical intermediate mechanism using a biotin-tyramide
98 reagent (**Figure 1A**) (*Hung et al., 2016; Martell et al., 2016*). HRP has been targeted to specific
99 cell-surface proteins through antibody conjugation to label target proteins and their binding part-
100 ners (*Rees et al., 2015b*). More recently, HRP was used as a soluble cell surface labeler to identify
101 rapid cell surface proteome changes in response to insulin (*Li et al., 2021*). Genetically encoded,
102 membrane-targeted APEX2 and HRP have also permitted promiscuous labeling of proteins in spe-
103 cific cellular compartments, but these efforts required cellular engineering (*Hung et al., 2016; Li*
104 *et al., 2020a*). We sought to expand the use of these tools to biotinylate surface proteins of cells
105 without the need for cellular engineering, enabling the specific enrichment of surface-resident pro-
106 teins for mass spectrometry analysis.

107 The first approach we tested was to tether a DNA-APEX2 conjugate to the cell membrane through
108 a lipidated DNA anchor. Gartner and co-workers have shown lipidated DNA anchors can tether to-
109 gether molecules or even cells (*McGinnis et al., 2019; Weber et al., 2014*). Here the lipidated DNA
110 is first added to cells, then hybridized with a complementary strand of DNA conjugated to APEX2
111 (**Figure 1B**, left panel). To conjugate DNA to APEX2, we leveraged the single unpaired cysteine in
112 the protein for site-specific bioconjugation of the complementary DNA. We first reacted APEX2 with
113 DBCO-maleimide, after which the DBCO moiety was readily conjugated with azido-DNA. The kinet-
114 ics of coupling was monitored using LC-MS and the conjugate was purified by nickel column chro-
115 matography, yielding a single conjugated product (**Figure 1 - Figure supplement 2A**) that retained
116 full enzymatic function relative to unlabeled APEX2 (**Figure 1 - Figure supplement 2B**). Microscopy
117 was used to observe the colocalization of DNA-conjugated APEX2 to the membrane (**Figure 1C**).
118 This result was recapitulated using flow cytometry, indicating that this approach results in surface
119 tethering of APEX2, an important step towards the specific labeling of the cell surfaceome (**Figure**
120 **1 - Figure supplement 2C**).

121 To avoid the need for bioconjugation, we also tested a commercially available reagent where
122 the promiscuous biotinylation reagent HRP is conjugated to the lectin wheat germ-agglutinin (WGA) (**Figure**
123 **1B**, right panel). WGA-HRP is used regularly in the glycobiology and neuroscience fields to label cell
124 membranes for immuno-histochemistry and live cell imaging (*Mathiasen et al., 2017; Wang and*
125 *Miller, 2016*). This is an inexpensive and widely available tool that only requires the presence of
126 surface protein N-acetylglucosamine (GlcNAc) and sialic acid glycans to localize HRP to the mem-
127 brane. The successful and rapid colocalization of WGA-HRP to the plasma membrane compared to
128 HRP alone was verified using immunocytochemistry, indicating this approach is a potential alter-
129 native for cell surface labeling (**Figure 1D**). Further testing showed that adding WGA-HRP to cells in

Figure 1. Direct labeling of promiscuous biotinylators to the cell membrane for rapid cell surface proteome characterization of small-scale biological samples. (A) Outline of enzymatic reaction mechanism. APEX2 and HRP both require biotin tyramide and hydrogen peroxide to produce the biotin-radical intermediate. (B) Tethering either enzyme is completed through differing mechanisms: (i) APEX2 is tethered through bio-conjugation of a single-strand of DNA, which is complementary to an exogenously added sequence of lipidated-DNA attached to the membrane, (ii) Commercially available wheat germ agglutinin (WGA)-HRP associates with native GlcNAc and sialic acid glycan moieties on cell surface proteins. (C) Microscopy images depicting the localization of DNA-APEX2 to the cell surface of KP-4 cells after introduction of the lipidated-DNA complementary strands. (D) Microscopy images depicting the localization of WGA-HRP to the membrane of KP-4 cells. All microscopy images are representative of two biological replicates.

Figure 1-Figure supplement 1. Expression, purification, and validation of APEX2 enzyme.

Figure 1-Figure supplement 2. Labeling and efficacy of APEX2 with DNA.

Figure 1-Figure supplement 3. WGA-HRP pre-incubation time on cells has no effect on labeling efficiency.

130 the presence hydrogen peroxide and biotin-tyramide led to robust surface labeling even with no
131 pre-incubation time (**Figure 1 - Figure Supplement 3**).

132 **Cell-tethered biotinylators more effectively label the surfaceome than non-tethered** 133 **biotinylators and are comparable to biocytin hydrazide**

134 Next, we set out to optimize labeling conditions for small-scale sample characterization. As APEX2
135 is kinetically slower than HRP (*Lam et al., 2015*), we used APEX2 to establish a suitable concentration
136 range of enzyme for cell surface labeling. We found that 0.5 μ M APEX2 produced maximal label-
137 ing of cells (**Figure 2 - Figure supplement 1A**) and maintained equivalent labeling across a range
138 of cell numbers (2.5e5 – 1e6 cells; **Figure 2 - Figure supplement 1B**). Next, we compared the effi-
139 ciency of DNA-APEX2, WGA-HRP, and their non-tethered counterparts to biotinylate a small sample
140 of 5e5 Expi293 cells. We found a 5- to 10-fold increase in biotin labeling for both tethered DNA-
141 APEX2 and WGA-HRP relative to non-tethered controls as assessed by flow cytometry (**Figure 2A**)
142 and western blotting (**Figure 2B**). Moreover, tethered DNA-APEX2 and WGA-HRP systems exhibited
143 similar biotinylation efficiency, suggesting either system is suitable for small-scale surfaceomics.
144 Having both systems is useful, as some cells may not widely express glycoproteins recognized by
145 commercially available lectin-HRP conjugates—such as some prokaryotic species—and therefore
146 could require the glycan-agnostic DNA-tethered APEX2 construct (*Schäffer and Messner, 2017*).

147 To compare the degree of surface protein enrichment these two systems offer, we enriched
148 biotinylated proteins generated with either approach and compared the resulting enrichments
149 using LC-MS/MS. As an initial efficacy comparison, cell surface labeling with DNA-labeled APEX2
150 or WGA-HRP was compared using 5e5 cells. In order to eliminate the possibility of suspension
151 cell-specific results, we used a popular cell line model of pancreatic cancer, KP-4. We observed
152 that the WGA-HRP identified slightly more plasma membrane annotated proteins in the Uniprot
153 Gene Ontology Cellular Component Plasma Membrane (GOCC-PM) database (>2 unique peptides,
154 found in all replicates) relative to DNA-APEX2, totaling 501 and 467, respectively. Notably, the
155 number of IDs for both cell-tethered enzymes was higher than their untethered counterparts, with
156 HRP identifying 389 cell surface proteins and APEX2 identifying 247 (**Figure 2C**). Importantly, in
157 the upset plot shown, the group with the highest intersection includes all four enzyme contexts,
158 showcasing the reproducibility of labeling through a similar free-radical based mechanism. The cell-
159 tethered biotinylators also showed heightened surface enrichment compared to their untethered
160 counterparts, as illustrated by the higher overall intensities for proteins annotated to the plasma
161 membrane (**Figure 2 - Figure supplement 2A and 2B**). As equal amounts of total protein is injected
162 on the LC-MS/MS instrument, the higher intensities for plasma membrane proteins suggest that
163 localizing the enzyme to the membrane increases labeling of the membrane compartment, which
164 we have previously observed with other enzymatic reactions (*Weeks et al., 2021*).

165 As the mode of tethering WGA-HRP involves GlcNAc and sialic acid glycans, we wanted to deter-
166 mine whether there was a bias towards Uniprot annotated 'Glycoprotein' vs 'Non-Glycoprotein' sur-
167 face proteins identified across the WGA-HRP, APEX2-DNA, APEX2, and HRP labeling methods. We
168 looked specifically at surface annotated proteins found in the SURFY database, which is the most
169 stringent surface protein database and requires that proteins have a predicted transmembrane
170 domain (*Bausch-Fluck et al., 2018*). We performed this analysis by measuring the average MS1
171 intensity across the top three peptides (LFQ area) for SURFY glycoproteins and non-glycoproteins
172 for each sample and dividing that by the total LFQ area found across all GOCC-PM annotated pro-
173 teins detected in each sample. We found similar normalized areas of non-glycosylated surface
174 proteins across all samples (**Figure 2 - Figure supplement 3**). If a bias existed towards glyco-
175 sylated proteins in the WGA-HRP compared to the glycan agnostic APEX2-DNA sample, then we
176 would have seen a larger percentage of non-glycosylated surface proteins identified in APEX2-DNA
177 over WGA-HRP. Due to the large labeling radius of the HRP enzyme, we find it unsurprising that the
178 WGA-HRP method is able to capture non-glycosylated proteins on the surface to the same degree
179 (*Rees et al., 2015b*). There is a slight increase in the area percentage of glycoproteins detected in

Figure 2. Membrane-localized peroxidases increases membrane proteome biotinylation compared to non-tethered counterparts. (A) Biotinylation of Expi293 cells treated with free enzyme (APEX2 or HRP) or cell-tethered enzyme (DNA-APEX2 or WGA-HRP) shown by flow cytometry. Signal is read out using Streptavidin-AlexaFluor-647. (B) Comparison of cell labeling with either free enzyme (APEX2 or HRP) or cell-tethered enzyme (DNA-APEX2 or WGA-HRP) shown by Streptavidin-800 western blot and total protein stain. Normalized area is plotted to the right. (C) Number of cell membrane proteins identified by mass spectrometry (>2 unique peptides, <1% FDR, found in both biological replicates) after treating 500,000 KP-4 pancreatic cancer cells with either free enzyme (APEX2 or HRP) or cell-tethered enzyme (DNA-APEX2 or WGA-HRP). (D) Microscopy images depicting extent of labeling with free HRP compared to WGA-HRP with and without the blocking sugar GlcNAc. All western blot images, microscopy images, mass spectrometry data, and flow cytometry data are representative of two biological replicates.

Figure 2-Figure supplement 1. Optimization of APEX2 concentrations on cell by flow cytometry.

Figure 2-Figure supplement 2. Rank ordered intensities for surface annotated proteins detected in tethered and untethered enzyme samples.

Figure 2-Figure supplement 3. Comparison of enrichment for glycosylated and non-glycosylated proteins.

Figure 2-Figure supplement 4. Total plasma membrane protein identifications for DNA-APEX2 and WGA-HRP labeling experiments as a function of time.

Figure 2-Figure supplement 5. WGA-HRP labeling is N-acetyl-D-glucosamine (GlcNAc) dependent.

Figure 2-Figure supplement 6. WGA-HRP can be used to label adherent cells on-plate.

Figure 2-source data 1. Uncropped western blots.

Figure 2-source data 2. Mass spectrometry analysis results table.

the WGA-HRP compared to the APEX2-DNA sample, but this is likely due to the fact that a greater number of surface proteins in general are detected with WGA-HRP. As HRP is known to have faster kinetics compared to APEX2, it was anticipated that WGA-HRP would outperform DNA-APEX2 in cell surface protein identifications. The heightened labeling of WGA-HRP was consistent with every cell line tested, including another pancreatic cancer model, PaTu8902, which resulted in more proteins identified in the WGA-HRP sample over DNA-APEX2 for both 1 and 2 minute time points (**Figure 2 - Figure supplement 4**).

To confirm that the improved labeling by WGA-HRP was due to the binding of sugar units on the cell surface, we performed a sugar-blocking experiment with WGA-HRP using N-acetyl-D-glucosamine (GlcNAc) that would block the conjugate from binding to the cell. By pre-incubating WGA-HRP with excess GlcNAc, the ability of WGA-HRP to label the cell surface was markedly lower than WGA-HRP without GlcNAc as observed by microscopy (**Figure 2D**). A similar effect was also seen by flow cytometry (**Figure 2 - Figure supplement 5**). In addition, we also tested an on-plate protocol for simpler cell surface labeling of adherent KP-4 cells. We showed that cell surface labeling in this manner was comparable to labeling when the cells were in suspension (**Figure 2 - Figure supplement 6**).

As WGA-HRP consistently outperformed DNA-APEX2 by proteomics and represents a more facile method amenable to broad application in the field, we chose to compare the proteomic labeling results of WGA-HRP to other standard cell surface labeling methods (sulfo-NHS-LC-LC-biotin and biocytin hydrazide) on a prostate epithelial cell line, RWPE-1 with and without oncogenic c-Myc overexpression. Sulfo-NHS-LC-LC-biotin reacts with primary amines to form amide conjugates, but has notoriously high background contamination with intracellular proteins (*Weekes et al., 2010*). Biocytin hydrazide labeling is a two-step process that first involves oxidizing vicinal diols on glycoproteins at the cell surface, then reacting the aldehyde byproducts with biocytin hydrazide (*Elschenbroich et al., 2010*). Both WGA-HRP and biocytin hydrazide had similar levels of cell surface enrichment on the peptide and protein level when cross-referenced with the SURFY curated database for extracellular surface proteins with a predicted transmembrane domain (**Figure 3 - Figure supplement 1A**). Sulfo-NHS-LC-LC-biotin and whole cell lysis returned the lowest percentage of cell surface enrichment, suggesting a larger portion of the total sulfo-NHS-LC-LC-biotin protein identifications were of intracellular origin, despite the use of the cell-impermeable format. These same enrichment levels were seen when the datasets were searched with the curated GOCC-PM database, as well as Uniprot's entire human proteome database (**Figure 3 - Figure supplement 1B**). Of the proteins quantified across all four conditions, biocytin hydrazide and WGA-HRP returned higher overall intensity values for SURFY-specified proteins than either sulfo-NHS-LC-LC-biotin or whole cell lysis. Importantly, although biocytin hydrazide shows slightly higher cell surface enrichment compared to WGA-HRP, we were unable to perform the comparative analysis at 500,000 cells—instead requiring 1.5 million—as the protocol yielded too few cells for analysis. All three methods were highly reproducible across replicates (**Figure 3 - Figure supplement 2A-C**). Compared to existing methods, WGA-HRP not only labels cells efficiently with much lower input material requirements, it is also able to enrich for cell surface proteins to a similar extent in a fraction of the time.

WGA-HRP identifies surface markers of Myc-driven prostate cancer in both cells and small EVs

Prostate cancer remains one of the most common epithelial cancers in the elderly male population, especially in Western nations (*Litwin and Tan, 2017; Rawla, 2019*). While metastatic progression of prostate cancer has been linked to many somatic mutations and epigenetic alterations (PTEN, p53, Myc etc.), more recent work determined that alterations in Myc occurs in some of the earliest phases of disease, i.e. in tumor-initiating cells (*Koh et al., 2010*). This finding promotes the idea that the development of early-stage diagnostic tools that measure these Myc-driven disease manifestations could improve detection and overall patient disease outcomes (*Koh et al., 2010; Rebello et al., 2017*). One mode of early detection that has gained prominence is the use of prostate cancer-

230 derived exosomes in patient serum and urine (*Duijvesz et al., 2013; McKiernan et al., 2016*). Small
231 EVs are known to play important roles in the progression of prostate cancer, including increasing
232 tumor progression, angiogenesis, metastasis, and immune evasion, making this subcellular parti-
233 cle an extremely informative prognostic tool for disease progression (*Akoto and Saini, 2021; Lorenc
234 et al., 2020; Saber et al., 2020*).

235 To elucidate promising targets in Myc induced prostate cancer, we utilized our WGA-HRP method
236 to biotinylate cells from both normal epithelial prostate cells (RWPE-1 Control) and oncogenic Myc-
237 induced prostate cancer cells (RWPE-1 Myc, **Figure 3A**). Importantly, by using an isogenic system,
238 we are able to delineate specific Myc-driven protein expression changes, which could be helpful in
239 the identification of non-invasive, early-detection diagnostics for cancer driven by early Myc induc-
240 tion. In addition to having marked overexpression of c-Myc in the RWPE-1 Myc cells compared to
241 Control, they also grow with a more mesenchymal and elongated morphology compared to their
242 Control cell counterparts (**Figure 3B**), which would suggest large cell surface changes upon onco-
243 genic Myc induction. We initially used WGA-HRP to quantitatively compare the cell surface profiles
244 of Myc-induced prostate cancer to Control cells and found large and bidirectional variations in their
245 surfaceomes (**Figure 3C, 3D**). We have highlighted the 15 most upregulated proteins in each cell
246 type that are annotated as extracellular surface proteins in the GOCC-PM database. Proteins that
247 are also found in the most restrictive SURFY database that requires a predicted transmembrane
248 domain are bolded in the figure. Proteins annotated to be secreted (Uniprot) from the cell are
249 italicized (*Bausch-Fluck et al., 2018*). All significantly ($p < 0.05$) upregulated SURFY and secreted
250 proteins (>2 -fold) are listed with corresponding fold-change quantification (**Figure 3 - Source data
251 3**). Vimentin, a marker known to be associated with epithelial-to-mesenchymal transition (EMT)
252 showed heightened expression, in the context of oncogenic Myc, as well as CDH2 (N-Cadherin),
253 another marker of EMT (**Figure 3D**) (*Liu et al., 2015; Nakajima et al., 2004*). While vimentin has
254 traditionally been described as an intracellular protein, an extracellular membrane-bound form
255 has been found to be important in the context of cancer (*Mitra et al., 2015; Noh et al., 2016*). AN-
256 PEP and fibronectin-1 were also highly upregulated. Notably, a number of HLA molecules were
257 downregulated in the Myc induced RWPE cells, consistent with prior findings of loss of MHC class I
258 presentation in prostate cancer (*Blades et al., 1995; Cornel et al., 2020; Dhatchinamoorthy et al.,
259 2021*). A subset of these findings were verified by both western blot (**Figure 3E**) and microscopy
260 (**Figure 3F**), which highlights the robustness of the protein quantification afforded by using this
261 method.

262 Next, we wanted to use our WGA-HRP method to quantify cell surface proteins on a sucrose-
263 gradient purified population of small EVs derived from both normal epithelial prostate cells (RWPE-
264 1 Control) and oncogenic Myc-induced prostate cancer cells (RWPE-1 Myc, **Figure 4A**). While su-
265 crose gradient centrifugation generally yields a mixture of vesicle populations, we wanted to con-
266 firm that our preparation enriched for vesicles originating from multiple vesicular bodies (MVBs),
267 consistent with an exosome-enriched sample (*Mathieu et al., 2021*). To do so, we prepared EVs
268 from both Control and Myc cells and carried out label-free quantification (LFQ) mass spectrome-
269 try on the whole EV lysates. After normalizing for cell number, we found the Myc cells produced
270 nearly 40% more EVs than the corresponding control cells, which is consistent with previous work
271 that has shown Myc overexpression yields higher quantities of EVs (*Kilinc et al., 2021*). After aver-
272 aging the intensities between Control and Myc derived EVs, many of the highest intensity proteins
273 (CD9, SDCB1, CD81, LAMP1, LAMP2, ALIX, and CD63) are consistent with MVB-derived vesicle bio-
274 genesis, supporting that the sample was likely enriched in EVs rather than other sedimentable
275 particles that can co-isolate during centrifugation (**Figure 4B**). Due to the complex process and ex-
276 tensive washing involved in small EV isolation, many standard labeling methods are not amenable
277 for EV surface labeling. Using WGA-HRP, we are able to biotinylate the small EVs before the su-
278 crose gradient purification and isolation steps (**Figure 4C**). This delineated an important subset of
279 proteins that are differentially expressed under Myc induction, which could serve as interesting tar-
280 gets for early-detection in patient urine or serum (**Figure 4D**). All significantly upregulated SURFY

Figure 3. WGA-HRP identifies a number of enriched markers on Myc-driven prostate cancer cells. (A) Overall scheme for biotin labeling, and label-free quantification (LFQ) by LC-MS/MS for RWPE-1 Control and Myc over-expression cells. (B) Microscopy image depicting morphological differences between RWPE-1 Control and RWPE-1 Myc cells after 3 days in culture. (C) Volcano plot depicting the LFQ comparison of RWPE-1 Control and Myc labeled cells. Red labels indicate upregulated proteins in the RWPE-1 Control cells over Myc cells and green labels indicate upregulated proteins in the RWPE-1 Myc cells over Control cells. All colored proteins are at least 2-fold enriched in either dataset between four replicates (two technical, two biological, $p < 0.05$). (D) Heatmap of the 30 most upregulated transmembrane (bold) or secreted (italics) proteins in either RWPE-1 Control or Myc cells. Scale indicates intensity, defined as (LFQ Area - Mean LFQ Area)/Standard Deviation. (E) Table indicating fold-change of most differentially regulated proteins by LC-MS/MS for RWPE-1 Control and Myc cells. (F) Upregulated proteins in RWPE-1 Myc cells (Myc, ANPEP, Vimentin, and FN1) are confirmed by western blot. (G) Upregulated surface proteins in RWPE-1 Myc cells (Vimentin, ANPEP, FN1) are detected by immunofluorescence microscopy. The downregulated protein HLA-B by Myc over-expression was also detected by immunofluorescence microscopy. All western blot images and microscopy images are representative of two biological replicates. Mass spectrometry data is based on two biological and two technical replicates ($N = 4$).

Figure 3-Figure supplement 1. Comparison of surface enrichment between replicates for different mass spectrometry methods.

Figure 3-Figure supplement 2. Comparison of replicates for different mass spectrometry methods show that WGA-HRP has comparable reproducibility to NHS-Biotin and Hydrazide labeling.

Figure 3-source data 1. Uncropped western blots.

Figure 3-source data 2. Mass spectrometry analysis results table.

Figure 3-source data 3. List of proteins comparing enriched targets (>2 -fold) in Myc cells versus Control cells.

281 and secreted proteins (>1.5-fold) are listed with corresponding fold-change quantification (**Figure**
282 **4 - source data 4**). This subset included fibronectin-1 (FN1) and ANPEP (**Figure 4E**), which were
283 further validated by quantitative western blotting (**Figure 4F**). A subset of these targets display
284 similar phenotypic changes to the parent cell, suggesting that they could be biomarker candidates
285 for non-invasive indicators of disease progression. While certain proteins are shuttled to vesicle
286 compartments largely based off of the extent of expression in the parent cell (Control: IFITM3,
287 BST2, HLA-B, Myc: ANPEP, SLC38A5, FN1), remarkably some proteins are singled out for small EV
288 packaging, indicating a pronounced differential shuttling mechanism of the proteome between
289 cells and EVs. This pattern was recapitulated in both the RWPE-1 Control cells and corresponding
290 EVs, as well as the Myc cells and EVs, where the majority of markers were unique to either cellular
291 or EV origin (**Figure 4 - Figure supplement 1**). These protein targets are of extreme interest for
292 not only biomarker discovery, but also understanding the role of small EVs in secondary disease
293 roles, such as interfering with immune function or priming the metastatic niche (*Costa-Silva et al.,*
294 **2015**).

295 Due to the difficulty of proteomic characterization of vesicular populations, our current under-
296 standing of EV protein shuttling remains limited. Prior proteomic EV analysis has involved whole
297 EV preparations, which lacks a surface protein enrichment step (*Bandu et al., 2019; Bilen et al.,*
298 **2017; Hosseini-Beheshti et al., 2012**). Not only is whole EV lysate analysis less advantageous for
299 the specific identification of cell surface proteins on EVs, but it makes it impossible to compare
300 cellular and EV samples due to the inherent surface area-to-volume differences between cells and
301 the vesicles they produce (*Doyle and Wang, 2019; Santucci et al., 2019*). Our WGA-HRP method
302 allows us to compare surface proteins between small EV populations, as well as between small
303 EV and cell samples (**Figure 5A**). By principle component analysis (PCA), each sample separates by
304 oncogenic status and origin (**Figure 5B**). Indeed, when performing functional annotation for each
305 gene cluster defined by the PCA, 'extracellular exosome' and 'extracellular vesicle' are the highest
306 ranking annotation features differentiating the EVs from their parent cells (**Figure 5C**). Through
307 this comparison, we were able to delineate a host of proteins that were upregulated in EVs over
308 their parent cells and vice versa (**Figure 5D**). Notably, secreted proteins were more highly repre-
309 sented in the EV surface proteome compared to cells. A subset of proteins were highly upregu-
310 lated in the small EVs compared to parent cell, including ITIH4, MFGE8, TF, DSG1, TSPAN14, AZGP1,
311 and IGSF8, (**Figure 5E**) and a subset of the findings were validated by western blot (**Figure 5F**).
312 All significantly ($p < 0.05$) upregulated SURFY and secreted proteins (>2-fold) are listed with corre-
313 sponding fold-change quantification (**Figure 5 - source data 3**). The samples showed good overlap
314 between replicates across all four datasets, with cellular and EV samples clustering by origin and
315 oncogenic status (**Figure 5 - Figure supplement 1**). To our knowledge, this is the first experiment
316 to wholistically characterize the surface proteome of both small EVs and parental cells. These
317 data strongly suggest that protein triage into EVs is a controlled process, enabling only a subset
318 of the cell surface proteome to be shuttled to this important compartment. Our data shows that
319 there are a variety of pan-prostate-EV markers, notably lactadherin (MFGE8), serotransferrin (TF),
320 inter-alpha-trypsin inhibitor (ITIH4), immunoglobulin superfamily 8 (IGSF8), desmoglein-1 (DSG1),
321 tetraspanin-14 (TSPAN14), and zinc-alpha-2-glycoprotein (AZGP1) (**Figure 5D**), which do not seem
322 to be Myc-specific. Some of the pan-prostate EV targets in our data have previously been linked
323 to cancer-specific contexts, and we show here that they are also found on Control EVs (*Shimagaki*
324 *et al., 2019; Tutanov et al., 2020; Philley et al., 2017*). Our work suggests that these markers are
325 more broadly associated with small EVs, regardless of disease status, outlining an expanded set of
326 targets to probe these vital compartments.

327 Discussion

328 The importance of understanding and characterizing cellular and EV membrane compartments
329 is vital for improving our understanding of vesicle biogenesis. New, improved methodologies
330 amenable to small-scale and rapid surface proteome characterization are essential for continued

Figure 4. WGA-HRP identifies a number of enriched markers on Myc-driven prostate cancer EVs. (A) Workflow for small EV isolation from cultured cells. (B) Labeled proteins indicating canonical exosome markers (ExoCarta Top 100 List) detected after performing label-free quantification (LFQ) from whole EV lysate. The LFQ intensities were averaged for Control and Myc EVs, and the resulting protein list is graphed from least abundant to most abundant. (C) Workflow of EV labeling and preparation for mass spectrometry. (D) Heatmap of the 30 most upregulated proteins in either RWPE-1 Control or Myc EVs. Scale indicates intensity, defined as (LFQ Area - Mean LFQ Area)/Standard Deviation. (E) Table indicating fold-change of most differentially regulated proteins by LC-MS/MS for RWPE-1 Control and Myc cells. (F) Upregulated proteins in RWPE-1 Myc EVs (ANPEP and FN1) are confirmed by western blot. Mass spectrometry data is based on two biological and two technical replicates (N = 4). Due to limited sample yield, one replicate was performed for the EV western blot.

Figure 4-Figure supplement 1. Venn diagram comparing enriched targets (>2-fold) in Cells and EVs.

Figure 4-source data 1. Uncropped western blots.

Figure 4-source data 2. Whole EV mass spectrometry analysis results table.

Figure 4-source data 3. Mass spectrometry analysis results table.

Figure 4-source data 4. List of proteins comparing enriched targets (>1.5-fold) in Myc EVs versus Control EVs.

development in the areas of therapeutics, diagnostics, and basic research. We sought to develop a simple, rapid surface protein labeling approach that was compatible with small sample sizes, while remaining specific to the cell surface. We took advantage of fast peroxidase enzymes and either complementary lipidated DNA technology (DNA-APEX2) or the glycan binding moiety wheat germ agglutinin (WGA-HRP) and demonstrated that tethering was much more effective than soluble addition, with increases in protein identification of between 30-90%. Additionally, we compared WGA-HRP to the existing methods, sulfo-NHS-LC-LC-biotin and biocytin hydrazide. While these alternative methods are robust, they are unable to capture time-sensitive changes, and are either plagued by low selectivity/specificity (NHS-Biotin) (*Weekes et al., 2010*) or the requirement for large sample inputs (biocytin hydrazide).

There are many advantages of our new methods over the current cell surface labeling technologies. Compared to both sulfo-NHS-LC-LC-biotin and biocytin hydrazide, WGA-HRP experiments require 2 minutes instead of 30 or 120 minutes, respectively. It is also able to enrich cell surface proteins much more efficiently than sulfo-NHS-LC-LC-biotin labeling. Furthermore, NHS peptide isolation and preparation is complicated due to the reactivity of NHS chemistry towards free-amines, which blocks tryptic and LysC cleavages typically used in proteomics (*Chandler and Costello, 2016; Hacker et al., 2017*).

The hydrazide method is highly effective for enriching cell surface proteins, but it is challenging for small sample sizes, due to the two-step labeling process and cell loss from the oxidation step and extensive washing. Additionally, neither NHS-biotin nor biocytin hydrazide are able to capture short time points to encompass dynamic changes at the cell surface. Due to the fast kinetics of peroxidase enzymes (1-2 min), our approaches could enable kinetic experiments to capture rapid post-translational trafficking of surface proteins, such as response to insulin, certain drug treatments, T-cell activation and synapse formation, and GPCR activation (*Valitutti et al., 2010; Gupta et al., 2019; Li et al., 2021*). Another disadvantage of the hydrazide method is that it can only enrich for proteins that are glycosylated at the cell surface and it is estimated that 10-15% of cell surface proteins are not glycosylated (*Apweiler, 1999*). Glycosylation patterns also readily change during tumorigenesis, which can alter the quantification of glycan-based labeling methods, such as biocytin hydrazide (*Reily et al., 2019*). While the WGA-HRP method requires glycosylated proteins to be present to bind, it is still able to label non-glycosylated proteins nearby due to its large labeling radius. It is a possibility that certain cells may have low or uneven levels of glycosylation on their surfaces. In these cases, the DNA-APEX2 method can be utilized to obtain effective labeling. However, both these peroxidase-based methods require the presence of tyrosine residues (natural abundance 3.3%) to react with the biotin-tyramide radical, which is not equally abundant in all proteins (*Dyer, 1971*).

With the WGA-HRP method, we were able to compare the surfaceome of small EVs to parental cells for Myc-induced prostate cancer cells and identified proteins that were upregulated in Myc-induced cells and EVs, as well as proteins that were differentially shuttled between EVs and parental cells. We found a number of Myc specific markers in our study, which were enriched in both Myc EV and Myc Cell samples. These include ANPEP, SLC38A5, FN1, CDH13, VIM, and CA12. ANPEP is a membrane-bound ectopeptidase that degrades N-termini with neutral amino acids and was found 140-fold upregulated in the Myc-induced cell compared to the Control cell and 49-fold upregulated in the Myc-induced EV compared to Control EV. This peptidase has been associated with angiogenesis and cancer growth (*Guzman-Rojas et al., 2012; Sørensen et al., 2013; Wickström et al., 2011*). Recent studies have shown ANPEP is systematically upregulated on isogenic cell lines expressing proliferative oncogenes (*Leung et al., 2020; Martinko et al., 2018*) or in tubular sclerosis bladder cancers (*Wei et al., 2020*), suggesting it is commonly upregulated in cancers. The second most differentially expressed protein between the Myc and Control samples was SLC38A5 (23 and 73-fold upregulated in cells and EVs, respectively). SLC38A5 is a glutamine co-transporter and has previously been shown to be a downstream target of c-Myc in glutamine-addicted cancers. Moreover, given that SLC38A5-based glutamine transport leads to proton flux and intracellular alkalinization,

Figure 5. WGA-HRP identifies a number of EV-specific markers that are present regardless of oncogene status. (A) Matrix depicting samples analyzed during LFQ comparison—Control and Myc cells, as well as Control and Myc EVs. (B) Principle component analysis (PCA) of all four groups analyzed by LFQ. Component 1 (50.4%) and component 2 (15.8%) are graphed. (C) Functional annotation was performed for each gene cluster using DAVID Bioinformatics Resource 6.8 and the highest ranking annotation features for the EV-specific gene cluster are shown. (D) Heatmap of the 50 most upregulated proteins in either RWPE-1 cells or EVs. Proteins are listed in decreasing order of expression with the most highly expressed proteins in EVs on the far left and the most highly expressed proteins in cells on the far right. Averages from all four replicates of each sample type are graphed. Scale indicates intensity, defined as (LFQ Area - Mean LFQ Area)/Standard Deviation. Extracellular proteins with annotated transmembrane domains are bolded and annotated secreted proteins are italicized. (E) Table indicating fold-change of most differentially regulated proteins by LC-MS/MS for RWPE-1 EVs compared to parent cells. (F) Western blot showing the EV specific marker ITIH4, IGSF8, and MFGE8. Mass spectrometry data is based on two biological and two technical replicates (N = 4). Due to limited sample yield, one replicate was performed for the EV western blot.

Figure 5-Figure supplement 1. Heatmap comparison of biological and technical replicates of RWPE-1 Control/Myc cells and EVs.

Figure 5-source data 1. Uncropped western blots.

Figure 5-source data 2. Mass spectrometry analysis results table.

Figure 5-source data 3. List of proteins comparing enriched targets (>2-fold) in Control EVs versus Control cells and Myc EVs versus Myc cells.

382 overexpression of SLC38A5 has also been hypothesized to be a strategy for pH regulation in can-
383 cer cells that regularly experience intracellular acidification due to high glycolytic flux (*Bhutia and*
384 *Ganapathy, 2016; Wise et al., 2008*). Additionally, Fibronectin-1 (FN1) was also found to be upregu-
385 lated in Myc samples over Control samples (5 and 63-fold upregulated in cells and EVs, respectively),
386 and has been shown to drive all stages of tumorigenesis (*Wang and Hielscher, 2017*). Importantly,
387 FN1 provides an extracellular scaffold by which other matrix proteins can be deposited. Through
388 these interactions with matrix proteins and cell-associated integrins, FN1 regulates cellular fate
389 decisions, proliferation, and metastasis (*Efthymiou et al., 2020*).

390 While some proteins were present in both the EV and cellular samples, others were only found
391 enriched in Myc EVs. THBS1, also known as thrombospondin-1 was over 10-fold upregulated in
392 Myc EVs over Control EVs. Interestingly, this relationship was not found in the parent cells, which
393 suggests that THBS1 is differentially shuttled into oncogenic EVs. The role of this protein has newly
394 been associated with the growth and metastasis of glioblastoma and a potential serum prognostic
395 factor in myeloid leukemia (*Zhu et al., 2020; Daubon et al., 2019*). Moreover, using a model of
396 THBS1 overexpressing breast cancer, recent work has shown that exosomes laden with THBS1
397 promote cancer cell migration via disruption of the endothelial barrier (*Cen et al., 2019*).

398 Another such target is CD44, which was over 8-fold upregulated in the Myc EVs over Control
399 EVs. CD44 has long been known to drive cancer progression and aberrant cell signaling (*Chen*
400 *et al., 2018*). Recently, CD44 has also been found to be preferentially loaded into cancer-derived
401 exosomes and has been implicated in driving chemoresistance in a model of doxorubicin-treated
402 breast cancer (*Wang et al., 2020*). Similarly, it has been shown that exosome-mediated transfer of
403 CD44 from cells with high metastatic potential promoted migratory behavior in neighboring cells
404 with low metastatic potential (*Shen et al., 2021*). These targets delineate an important subset of
405 proteins that are triaged into EVs and could play long-range roles in promoting tumorigenesis and
406 downstream metastasis (*Costa-Silva et al., 2015; Demory Beckler et al., 2013; Hoshino et al., 2015;*
407 *Peinado et al., 2012*).

408 As research shifts into analyzing native biological samples from extracellular vesicles to xenograft
409 models or patient biopsies, it will become increasingly important to develop sensitive, effective
410 methods to label these small samples sizes. It is our hope that these tools will provide much
411 needed avenues by which to pursue pressing biological questions in the areas of diagnostic and
412 therapeutic development, as well as basic research.

Table 2. Key Resources Table

Reagent type (species)	Designation	Source	Identifiers	Additional information
Strain, strain background (E. coli)	BL21(DE3)pLysS	Promega	L1195	-
Cell line (human)	Expi293F	Thermo Scientific	A14527	-
Cell line (human)	PaTu8902	https://doi.org/10.7554/eLife.45313.001	-	-
Cell line (human)	KP-4	https://doi.org/10.7554/eLife.45313.001	-	-
Cell line (human)	RWPE-1	https://doi.org/10.1073/pnas.2018861118	-	Control and Myc overexpression lines
Antibody	anti-HisTag-650 (mouse monoclonal)	Invitrogen	MA1-21315-D650	1:100, flow cytometry, immunocytochemistry
Antibody	Biotin-conjugated anti-HRP (rabbit polyclonal)	Rockland	200-4638-0100	1:100, flow cytometry, immunocytochemistry
Antibody	anti-ANPEP (sheep polyclonal)	R & D Systems	AF3815	1:1000
Antibody	anti-Vimentin (rabbit monoclonal)	Cell Signaling Technology	57415	1:1000
Antibody	anti-FN1 (rabbit polyclonal)	Abcam	ab2413	1:1000
Antibody	anti-HLA-B (rabbit polyclonal)	Protein-Tech	17260-1-AP	1:1000
Antibody	anti-ITIH4 (rabbit polyclonal)	Atlas Antibodies	HPA003948	1:1000
Antibody	anti-MFGE8 (rabbit polyclonal)	Thermo Scientific	PAS-82036	1:1000
Antibody	anti-IGSF8 (goat polyclonal)	R & D Systems	AF3117-SP	1:1000
Antibody	Goat Anti-Rabbit HRP (goat polyclonal)	Thermo Scientific	31460	1:10000
Antibody	Rabbit Anti-Sheep HRP (rabbit polyclonal)	Thermo Scientific	31480	1:10000
Recombinant DNA reagent	APEX2	Twist Biosciences	-	-
Sequence-based reagent	DBCO-DNA (Conjugated to APEX2)	IDT	-	-
Sequence-based reagent	Lipid DNA (Anchor and Co-Anchor)	https://doi.org/10.1038/s41592-019-0433-8	-	-
Peptide, recombinant protein	HRP, Peroxidase	Thermo Fisher Scientific	31490	-
Peptide, recombinant protein	WGA, Peroxidase (WGA-HRP)	Vector Laboratories	PL-1026-2	-
Peptide, recombinant protein	IRDye 800CW Streptavidin	Li-Cor	926-32230	1:10000
Peptide, recombinant protein	IRDye 680RD Donkey anti-Goat IgG	Li-Cor	925-68074	1:10000
Commercial assay or kit	BCA assay	Thermo Scientific	23228	-
Commercial assay or kit	Preomics iST kit	PreOmics	P.O.00027	-
Commercial assay or kit	Pierce Quantitative Colorimetric Peptide Assay	Thermo Fisher Scientific	23275	-
Commercial assay or kit	Expi293 Media	Thermo Scientific	A1435101	-
Commercial assay or kit	Keratinocyte SFM Media (1X)	Thermo Scientific	17005042	-
Commercial assay or kit	MycAlert PLUS mycoplasma detection kit	Lonza	LT07-703	-
Chemical compound, drug	Trolox	Fisher Scientific	501176131	-
Chemical compound, drug	Sodium Ascorbate	Sigma-Aldrich	A4034-100G	-
Chemical compound, drug	N-acetyl-D-glucosamine	Sigma-Aldrich	A3286-5G	-
Chemical compound, drug	H ₂ O ₂ (1X)	Sigma-Aldrich	H1009-100ML	-
Chemical compound, drug	Hemin-Cl	Sigma-Aldrich	51280-1G	-
Chemical compound, drug	Maleimide DBCO	Click Chemistry Tools	A108P-10	-
Chemical compound, drug	Biotin Tyramide	Sigma-Aldrich	SML2135-50MG	-
Chemical compound, drug	Sodium Pyruvate	Sigma-Aldrich	S8636-100ML	-
Chemical compound, drug	Sodium Periodate	Sigma-Aldrich	311448-5G	-
Chemical compound, drug	Biocytin Hydrazide	Biotium	90060	-
Chemical compound, drug	Aniline	Sigma-Aldrich	242284	-
Chemical compound, drug	Protease Inhibitor Cocktail	Sigma-Aldrich	P8340-1ML	-
Chemical compound, drug	High Capacity Neutravidin Resin	Thermo Fisher Scientific	29204	-
Chemical compound, drug	poly-D-lysine	Thermo Scientific	A3890401	-
Chemical compound, drug	Sulfo NHS LC-LC Biotin	Thermo Scientific	A35358	-
Software, algorithm	FlowJo	FlowJo (https://www.flowjo.com)	RRID:SCR_008520	-
Software, algorithm	GraphPad Prism	Prism (https://www.graphpad.com/scientific-software/prism/)	RRID:SCR_002798	-
Software, algorithm	Morpheus	Morpheus (https://software.broadinstitute.org/morpheus/)	-	-
Software, algorithm	Fiji	Fiji (https://fiji.sc)	RRID:SCR_002285	-
Other	Penicillin/streptomycin	Thermo Fisher Scientific	15-140-122	-
Other	Fetal bovine serum	Gemini Bio-Products	100-106	-
Other	Trypsin (0.05%)	Life Technologies	25300054	-

Large-Scale APEX2 Expression, Purification, and Heme Reconstitution

APEX2 was expressed using previous methods in BL21(DE3)pLysS cells (*Howarth and Ting, 2008*). Briefly, APEX2 expression plasmid was transfected into competent BL21(DE3)pLysS cells and heat shocked for 45 seconds before being placed on ice. Cells were plated on LB/Carb plates and grown overnight at 37°C. A single colony was isolated and grown in a mixture of 30 ml of 2XYT + Carb overnight at 37°C while shaking. The overnight culture was combined with 3 L of 2XYT with Carb and placed in a 37°C shaking incubator. At an OD₆₀₀ of 0.6, 100 µg/ml of IPTG was added and the temperature of the incubator was lowered to 30°C. Cells were allowed to incubate for 3.5 hours and were spun down at 6,000xg for 20 minutes. Cell pellet was resuspended in protease inhibitor containing resuspension buffer (5 mM Imidazole, 300 mM NaCl, 20 mM Tris pH=8) and mixed thoroughly. The mixture was sonicated at 50% (5 seconds on:15 seconds off) for 5 minutes on ice to avoid bubble formation. Lysate was mixed by inversion at 4°C for 15 minutes and spun down at 19,000xg for 20 minutes. The slurry was introduced to 5 ml of washed Nickel resin slurry and allowed to bind by gravity filtration. The beads were washed 3x with wash buffer (30 mM Imidazole, 300 mM NaCl, 20 mM Tris pH=8) and eluted in 5 ml of elution buffer (250 mM Imidazole, 300 mM NaCl, 20 mM Tris pH=8) before undergoing buffer exchange into PBS.

Enzyme underwent heme reconstitution as per previous methods (*Cheek et al., 1999*). Briefly, 50 mg of hemin-Cl (Sigma) was diluted in 2.0 mL of 10 mM NaOH. The mixture was thoroughly resuspended, then diluted further using 8.0 mL of 20 mM KPO₄, pH 7.0, and vortexed extensively. Mixture was spun down at 4,000xg 2x to eliminate insoluble hemin. APEX2 was diluted 1:2 in 20 mM KPO₄. 6 ml of heme stock was added to 2 ml of APEX over 20 minutes and allowed to rotate at 4°C wrapped in tin foil for 3 hours. The mixture was introduced to a column with 20 ml of DEAE

436 Sepharose pre-equilibrated in 20 mM KPO₄, pH 7.0 buffer. Enzyme was eluted using 100 mM KPO₄
437 and spin concentrated. To verify complete reconstitution, absorbance was measured at 403 and
438 280 nm. A403/280 > 2.0 is considered sufficient for reconstitution. The isolated protein was flash
439 frozen and stored at -80°C for long-term storage. Each batch of enzyme was run out on a 4-12%
440 Bis-Tris gel to confirm purity (**Figure 1 - Figure supplement 1**).

441 **APEX2 DNA labeling protocol**

442 APEX2 was incubated at 50 μM with 40 molar equivalents of maleimide-DBCO for 5 hours at room
443 temperature in PBS. The reaction was desalted with Zeba columns (7 kDa cutoff). 2.5 molar equiv-
444 alents of Azido-DNA was added to the reaction and incubated at 4°C overnight. Successful conju-
445 gation was monitored by LC-MS before the mixture was purified by nickel column.

446 **Cell culture**

447 Prior to all experiments, cells were tested for the presence of mycoplasma (MycoAlert PLUS, Lonza,
448 LT07-703). Expi293 suspension cells were maintained in Expi293 media (Thermo, A1435101) while
449 rotating at 125 rpm in a 37°C incubator with 8% CO₂. Cells were split every 3 days by diluting into
450 new media. Adherent PaTu8902 and KP-4 cells were grown in pre-warmed Iscove's Modified Dul-
451 becco's Media (IMDM) supplemented with 10% FBS (Gemini Bio-Products, 100-106) and 5% Peni-
452 cillin/Streptomycin (Thermo Fisher Scientific, 15-140-122) at 37°C in a 5% CO₂-humidified incubator.
453 Adherent RWPE-1 prostate cells were grown in complete keratinocyte-SFM (Thermo; 17005-042)
454 supplemented with bovine pituitary extract (BPE), recombinant EGF, and 5% penicillin/streptomycin
455 at 37°C in a 5% CO₂-humidified incubator. The media was exchanged every two days. For splitting,
456 cells were lifted with 0.05% Trypsin (Life Technologies) and quenched with 5% FBS before spinning
457 down cells to remove residual trypsin and FBS. Cells were then plated in pre-warmed complete
458 keratinocyte-SFM media.

459 **Microscopy**

460 Cells were plated at a density of 15,000 cells per well in a 96-well clear bottom plate (Greiner Bio-
461 One, 655090) pre-treated with poly-D-lysine (Thermo Scientific, A3890401). Cells were allowed 48
462 hours to reattach and grow undisturbed. Cells were washed 3x in cold PBS. For DNA-APEX2, 100 μl
463 of 0.5 μM enzyme solution was combined with anchor and co-anchor at a final concentration of 1
464 μM. For all other enzymes, enzyme was combined with PBS at a final concentration of 0.5 μM. For
465 sugar blocking studies, 100 μl of diluted enzyme solution (0.5 μM) was combined with 100 mg/ml
466 N-acetyl-D-glucosamine (Sigma Aldrich, A3286-5G). Cells were allowed to sit on ice for 5 minutes
467 to allow WGA to bind fully, as labeling was not altered by increased incubation time (**Figure 1 -**
468 **Figure supplement 3**). Biotin tyramide (Sigma Aldrich, SML2135-50MG) was added to cells with a
469 final concentration of 500 μM before adding 1 mM of H₂O₂. Reaction was allowed to continue for
470 2 minutes before rinsing cells 3x with 1X quench buffer (10 mM sodium ascorbate + 5 mM Trolox +
471 1 mM sodium pyruvate). The cells were rinsed 2x with PBS and crosslinked with 4% PFA for 10 min-
472 utes at RT. Cells were washed 3x with PBS before introduction to 1:100 primary antibody. Primary
473 antibodies used were: HisTag-650 (Invitrogen, MA1-21315-D650), Streptavidin-488 (Thermo Fisher
474 Scientific, S-11223), biotin-conjugated anti-HRP (Rockland, 200-4638-0100), ANPEP (R&D Systems,
475 AF3815), vimentin (Cell Signaling Technology, 5741S), and HLA-B (ProteinTech, 17260-1-AP). Cells
476 were washed 3x in PBS and imaged on an IN Cell Analyzer 6500. Images were processed in Fiji
477 using the Bio Formats plugin (*Linkert et al., 2010; Schindelin et al., 2012*).

478 **Cell-tethered APEX2, soluble APEX2, cell-tethered WGA-HRP and soluble HRP cell** 479 **surface labeling**

480 Cultured cells were grown for 3 days in tissue culture plates and dissociated by addition of versene
481 (PBS + 0.05% EDTA). Cells were washed 3x in PBS (pH 6.5), resuspended in PBS (pH 6.5) and aliquoted
482 to 500,000 cells per sample. Samples were resuspended in 100 μL of PBS (pH 6.5). For anchored

483 APEX2 samples, lipidated anchor DNA was allowed to bind for 5 minutes at 1 μ M on ice, followed by
484 1 μ M of lipidated co-anchor DNA on ice for 5 minutes. 0.5 μ M DNA-labeled APEX2 was allowed to
485 bind on cells for 5 minutes before final wash with PBS (pH 6.5). For soluble APEX2, WGA-HRP, and
486 soluble HRP samples, cells were resuspended in 0.5 μ M of the corresponding enzyme. WGA-HRP
487 was allowed to bind to cells for 5 minutes on ice. Biotin tyramide was added at a final concentra-
488 tion of 500 μ M and mixed thoroughly, before the addition of 1 mM H₂O₂. Cells underwent labeling
489 in a heated shaker (500 rpm) at 37°C for 2 minutes before being quenched with 5 mM Trolox/10
490 mM Sodium Ascorbate/1 mM Sodium Pyruvate. Cells were washed 2x in quench buffer and spun
491 down. The pellet was either further processed for flow cytometry, western blot, or flash frozen in
492 liquid nitrogen for mass spectrometry.

493 **On plate WGA-HRP cell surface labeling**

494 KP-4 cells were grown on a 6 cm tissue culture treated plate and washed 3x with PBS (pH 6.5). 2 mL
495 of 0.5 μ M WGA-HRP in PBS (pH 6.5) was added to the plate, followed by biotin tyramide (0.5mM final
496 concentration) and H₂O₂ (1mM final concentration). After a 2 minute incubation at 37°C, the cells
497 were washed 2x with 5 mM Trolox/10 mM Sodium Ascorbate/1 mM Sodium Pyruvate quenching
498 solution. The cells were washed 1x with PBS before being lifted with versene (PBS + 0.05% EDTA).
499 Once lifted, the cells were washed once with PBS and subsequently processed for flow cytometry
500 analysis.

501 **Biocytin hydrazide cell surface labeling**

502 Cultured cells were grown for 3 days in tissue culture plates and dissociated by addition of versene
503 (PBS + 0.05% EDTA). Cells were washed 3x in PBS (pH 6.5), resuspended in PBS (pH 6.5) and aliquoted
504 to 1.5 million cells per sample. Samples were resuspended in 100 μ L of PBS (pH 6.5) and fresh
505 sodium periodate (Sigma Aldrich, 311448, 1 μ L of a 160 mM solution) was added to each sam-
506 ple. The samples were mixed, covered in foil, and incubated while rotating at 4°C for 20 minutes.
507 Following three washes with PBS (pH 6.5), the samples were resuspended in 100 μ L of PBS (pH
508 6.5) with the addition of 1 μ L of aniline (Sigma Aldrich, 242284, diluted 1:10 in water) and 1 μ L of
509 100 mM biocytin hydrazide (Biotium, 90060). The reaction proceeded while rotating at 4°C for 90
510 minutes. The samples were then washed 2x with PBS (pH 6.5) and spun down. The pellet was ei-
511 ther further processed for flow cytometry, western blot, or flash frozen in liquid nitrogen for mass
512 spectrometry.

513 **Sulfo-NHS-LC-LC-biotin cell surface labeling**

514 Cultured cells were grown for 3 days in tissue culture plates and dissociated by addition of versene
515 (PBS + 0.05% EDTA). Cells were washed 3x in PBS (pH 7.4), resuspended in PBS (pH 8) and aliquoted
516 to 1.5 million cells per sample. Samples were resuspended in 50 μ L of PBS (pH 8). An aliquot of
517 EZ-Link Sulfo-NHS-LC-LC-Biotin (ThermoFisher, 21338) was resuspended in 150 μ L of PBS (pH 8).
518 7.5 μ L was added to each cell sample and the reaction proceeded while rotating at 4°C for 30
519 minutes. The reaction was quenched by the addition of 2.5 μ L of 1M Tris (pH 8.0). The samples
520 were washed 2x in PBS (pH 8.0) and spun down. The pellet was either further processed for flow
521 cytometry, western blot, or flash frozen in liquid nitrogen for mass spectrometry.

522 **Flow cytometry for cell surface biotinylation**

523 After labeling and quench washes, cells were washed once with PBS + 2% BSA to inhibit nonspe-
524 cific binding. Samples were then incubated with 100 μ L Streptavidin-Alexa Fluor 647 (Thermo Fis-
525 cher, 1:100 in PBS + 2% BSA). Following a 30-minute incubation at 4°C while rocking, samples were
526 washed three times with PBS + 2% BSA. Samples were analyzed in the APC channel and quantified
527 using a CytoFLEX (Beckman Coulter). All flow cytometry data analysis was performed using FlowJo
528 software.

529 **RWPE-1 small EV isolation and labeling protocol**

530 RWPE-1 Control and Myc cells were plated at 7 million and 4 million cells per plate, respectively,
531 across 16 x 15 cm² plates and allowed to grow in normal keratinocyte-SFM media with provided
532 supplements. Small EVs were isolated as previously described (*Poggio et al., 2019*). Briefly, two
533 days prior to EV isolation, media was replaced with 15 milliliters BPE-free keratinocyte-SFM media.
534 For vesicle enrichment, media was isolated after two days in BPE-free media and centrifuged at 300
535 x g for 10 minutes at RT, followed by 2,000 x g for 20 minutes at 4°C. Large debris was cleared by a
536 12,000 x g spin for 40 minutes at 4°C. The pre-cleared supernatant was spun a final time at 100,000
537 x g at 4°C for 1 hr to pellet extracellular vesicles. Isolated extracellular vesicles were brought up
538 in 50 µl of PBS with 0.5 µM of WGA-HRP and the mixture was allowed to bind on ice for 5 min-
539 utes. WGA-HRP bound vesicles were placed on a shaker (500 rpm) at 37°C before the addition of
540 biotin tyramide (0.5 mM final concentration) and H₂O₂ (1 mM final concentration). Vesicles under-
541 went labeling for 2 minutes before being quenched with 5 mM Trolox/10 mM Sodium Ascorbate/1
542 mM Sodium Pyruvate. Biotinylated small EVs were purified from other sedimentable particles by
543 further centrifugation on a sucrose gradient (20-60%) for 16 hours at 4°C at 100,000xg. Precisely,
544 the gradient was loaded using 0%, 20%, 40%, and 60% sucrose fractions from top to bottom. The
545 sample was loaded at the bottom in 60% sucrose and the purified small EVs were isolated in the
546 20-40% sucrose fractions. Differential sucrose centrifugation yielded between 3-5 µg of small EVs.

547 **Western blot protocol**

548 Cultured cells were grown in 15 cm² tissue culture plates and dissociated by addition of versene
549 (PBS + 0.05% EDTA). Cells were washed in PBS (pH 6.5) and resuspended in 100 µl PBS (pH 6.5) at
550 a concentration of 10 million cells/ml in PBS (pH 6.5). Cells were labeled, reaction was quenched
551 with 1X NuPage Loading Buffer, and immediately boiled for 5 minutes. To enable proper addition
552 of lysate to gel wells, the mixture was thinned with addition of nuclease, and the disulfides were
553 reduced with BME. The samples were subjected to electrophoresis in a 4-12% NuPage Gel until
554 the dye front reached the bottom of the gel cast. For cell and EV blots, equal amounts of protein
555 content quantified by BCA assay were prepared in 1X NuPage Loading Buffer with BME and boiled
556 for 5 minutes. Samples were loaded and subjected to electrophoresis in a 4-12% NuPage Gel until
557 the dye front reached the bottom of the gel cast. Prepared gels were placed in iBlot2 transfer stacks
558 and transferred using the P0 setting on the iBlot 2 Gel Transfer Device. The PVDF membrane was
559 blocked in TBS Odyssey Blocking buffer for 1 hour at RT. Membranes were washed in TBST and
560 incubated with Streptavidin-800 (1:10,000 dilution, Licor, 926-32230) for 30 minutes in TBS Odyssey
561 Blocking buffer + 0.1% Tween 20. Membranes were washed in TBST 3x with a final wash in water.
562 Membranes were visualized using an Odyssey DLx imager. Western blot samples were run and
563 quantified 2-3 times and a representative image was displayed in figures.

564 For cell and EV blots, equal amounts of protein content quantified by BCA assay was prepared
565 in 1X NuPage Loading Buffer with BME and boiled for 5 minutes. Samples were loaded and sub-
566 jected to electrophoresis in a 4-12% NuPage Gel until the dye front reached the bottom of the gel
567 cast. Prepared gels were placed in iBlot2 transfer stacks and transferred using the P0 setting on
568 the iBlot 2 Gel Transfer Device. The PVDF membrane was blocked in TBS Odyssey Blocking buffer
569 for 1 hour at RT. Membranes were washed in TBST and incubated overnight in primary antibody at
570 4°C in TBS Odyssey Blocking buffer + 0.1% Tween 20 while shaking. Primary antibodies used were
571 ANPEP (R&D Systems, AF3815), FN1 (Abcam, ab2413), vimentin (Cell Signaling Technology, 5741S),
572 ITIH4 (Atlas Antibodies, HPA003948), MFGE8 (Thermo Scientific, PA5-82036), IGSF8 (R&D Systems,
573 AF3117-SP). Membranes were washed in 3x TBST before introduction to a 1:10,000 dilution of sec-
574 ondary antibody in TBS Odyssey Blocking buffer + 0.1% Tween 20 for 1 hour at room temperature
575 while shaking. Secondary antibodies used were Goat Anti-Rabbit HRP (Thermo Scientific, 31460)
576 and Rabbit Anti-Sheep HRP (Thermo Scientific, 31480). Blots were imaged after 5 minutes in the
577 presence of SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific,
578 34577) and imaged using a ChemiDoc XRS+. Western blot samples were run and quantified 2-3

579 times and a representative image was displayed in figures. EV blot was run once due to limited
580 sample availability. EVs derived for western blotting were cultured and harvested independently
581 of either biological replicate used for mass spectrometry analysis.

582 **Proteomic preparation for whole EVs**

583 Whole EV pellets were previously flash frozen after collection. EVs were processed for LC-MS/MS
584 using a PreOmics iST kit (P.O.00027). Briefly, EV pellets were brought up in 50 µl of provided LYSE
585 solution and boiled with agitation for 10 minutes. The provided enzymes mixture (Trypsin and LysC)
586 were resuspended in 210 µl of RESUSPEND buffer, mixed, and added to the lysed EVs. Samples
587 were allowed to mix at 500 rpm for 1.5 hours at 37°C, before being quenched with 100 µl of STOP
588 solution. Sample was spun in provided C18 spin cartridge and washed 1X with 200µl of WASH 1 and
589 WASH 2. Peptides were eluted with 2X 100 µl of ELUTE, dried, and resuspended with the provided
590 LC-LOAD solution. Peptides were quantified using Pierce Quantitative Colorimetric Peptide Assay
591 (Thermo Fisher Scientific, 23275).

592 **Proteomic preparation for surface enriched samples**

593 Frozen cell and EV pellets were lysed using 2X RIPA buffer (VWR) with protease inhibitor cocktail
594 (Sigma-Aldrich; St. Louis, MO) at 4°C for 30 mins. Cell lysate was then sonicated, clarified, and
595 incubated with 100 µl of neutravidin agarose slurry (Thermo Fisher Scientific, 29204) at 4°C for 1
596 hr. The bound neutravidin beads were washed in 2 ml Bio-spin column (Bio-Rad, 732-6008) with 5
597 ml RIPA buffer, 5 ml high salt buffer (1M NaCl, PBS pH 7.5), and 5 ml urea buffer (2M urea, 50mM
598 ammonium bicarbonate) to remove non-specific proteins. Beads were allowed to fully drain before
599 transferring to a Low-bind Eppendorf Tube (022431081) with 2M Urea. Sample was spun down at
600 1,000xg and aspirated to remove excess liquid. Samples were brought up in 100 µl of 4M Urea
601 digestion buffer (50 mM Tris pH 8.5, 10 mM TCEP, 20 mM IAA, 4 M Urea) and allowed to reduce
602 and alkylate for 10 mins at 55°C while shaking. After the addition of 2 µg of total reconstituted
603 Trypsin/LysC, the sample was incubated for 2 hours at RT. To activate the trypsin, mixture was
604 diluted with 200 µl of 50 mM Tris pH 8.5 to a final Urea concentration of below 1.5 M. The mixture
605 was covered and allowed to incubate overnight at RT. The mixture was isolated from the beads by
606 centrifugation in a collection column (Pierce; 69725) before being acidified with 10% TFA until pH
607 of 2.0 was reached. During this time, a Pierce C18 spin column (Pierce, 89873) was prepared as
608 per manufacturing instructions. Briefly, C18 resin was washed twice with 200 µl of 50% LC-MS/MS
609 grade ACN. The column was equilibrated with two 200µl washes of 5% ACN/0.5% TFA. The pre-
610 acidified sample was loaded into the C18 column and allowed to fully elute before washing twice
611 with 200 µl washes of 5% ACN/0.5% TFA. One final wash of 200 µl 5% ACN/1% FA was done to
612 remove any residual TFA from the elution. Samples were eluted in 70% ACN, dried, and dissolved
613 in 0.1% formic acid, 2% acetonitrile prior to LC-MS/MS analysis. Peptides were quantified using
614 Pierce Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific, 23275).

615 **LC-MS/MS**

616 Liquid chromatography and mass spectrometry was performed as previously described (*Meier*
617 *et al., 2020*). Briefly, approximately 200 ng of peptides were separate using a nanoElute UHPLC
618 system (Bruker) with a pre-packed 25 cm x 75 µm Aurora Series UHPLC column + CaptiveSpray
619 insert (CSI) column (120 Å pore size, IonOpticks, AUR2-25075C18A-CSI) and analyzed on a timsTOF
620 Pro (Bruker) mass spectrometer. Peptides were separated using a linear gradient of 2-34% solvent
621 B (Solvent A: 2% acetonitrile, 0.1% formic acid, solvent B: acetonitrile, 0.1% formic acid) over 100
622 mins at 400 nL/min. Data-dependent acquisition was performed with parallel accumulation-serial
623 fragmentation (PASEF) and trapped ion mobility spectrometry (TIMS) enabled with 10 PASEF scans
624 per topN acquisition cycle. The TIMS analyzer was operated at a fixed duty cycle close to 100% using
625 equal accumulation and ramp times of 100 ms each. Singly charged precursors were excluded by
626 their position in the m/z-ion mobility plane, and precursors that reached a target value of 20,000

arbitrary units were dynamically excluded for 0.4 min. The quadrupole isolation width was set to 2 m/z for m/z < 700 and to 3 m/z for m/z > 700 and a mass scan range of 100-1700 m/z. TIMS elution voltages were calibrated linearly to obtain the reduced ion mobility coefficients (1/K0) using three Agilent ESI-L Tuning Mix ions (m/z 622, 922 and 1,222).

Data Processing and Analysis

Briefly, for general database searching, peptides for each individual dataset were searched using PEAKS Online X version 1.5 against both the plasma membrane (PM) annotated human proteome (Swiss-prot GOCC database, August 3, 2017 release) and the entire Swiss-prot Human Proteome (Swiss-prot). We acknowledge the identification of a number of proteins not traditionally annotated to the plasma membrane, which were published in the final Swiss-prot GOCC-PM database used. Additionally, to not miss any key surface markers such as secreted proteins or anchored proteins without a transmembrane domain, we chose to initially avoid searching with a more stringent protein list, such as the curated SURFY database. However, following the analysis we bolded proteins found in the SURFY database and italicized proteins known to be secreted (Uniprot). Enzyme specificity was set to trypsin + LysC with up to two missed cleavages. Cysteine carbamidomethylation was set as the only fixed modification; acetylation (N-term) and methionine oxidation were set as variable modifications. The precursor mass error tolerance was set to 20 PPM and the fragment mass error tolerance was set to 0.05 Da. Data was filtered at 1% for both protein and peptide FDR and triaged by removing proteins with fewer than 2 unique peptides. All mass spectrometry database searching was based off of two biological replicates. Biological replicates underwent washing, labeling, and downstream LC-MS/MS preparation separately.

For comparative label-free quantification of cellular and EV samples, datasets were searched using PEAKS Online X version 1.5 against the plasma membrane (PM) annotated human proteome (Swiss-prot GOCC database, August 3, 2017 release). Enzyme specificity was set to trypsin + LysC with up to two missed cleavages. Cysteine carbamidomethylation was set as the only fixed modification; acetylation (N-term) and methionine oxidation were set as variable modifications. The precursor mass error tolerance was set to 20 PPM and the fragment mass error tolerance was set to 0.05 Da. Data was filtered at 1% for both protein and peptide FDR and triaged by removing proteins with fewer than 2 unique peptides. Label free quantification of protein was completed by taking the average intensity of the top three most intense peptides for each protein. Data was normalized by total area sum intensity for each sample. Using Perseus, all peak areas were log2(x) transformed and missing values were imputed separately for each sample using the standard settings (width of 0.3, downshift of 1.8). Significance was based off of a standard unpaired Student t test with unequal variances across all four replicates. Reported peak area values represent the averages of all four replicates—two biological and two technical replicates. For representation of the data in figures, a Z-score was computed and is defined as (LFQ Area - Mean LFQ Area)/Standard Deviation. Protein IDs that were not annotated to be secreted or expressed extracellularly were removed. Further, in the Cell versus EV graph, any proteins that showed a standard deviation (SD) greater than 1.5 between Control and Myc of each sample type (EV or Cell) were removed to avoid representation of oncogene-specific changes. Oncogene-specific changes are instead shown in **Figure 3 - source data 3, Figure 4 - source data 4, and Figure 5 - source data 3**. EVs and cells from different biological replicates were cultured on different days. Desalting, quantification, and LC-MS/MS runs were performed together. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (*Perez-Riverol et al., 2019*) partner repository with the dataset identifier PXD028523.

To compare replicates of data in **Figure 3 - Figure supplement 2**, a simple linear regression was performed on total area sum intensity normalized data. Replicate 1 was graphed against Replicate 2 for biocytin hydrazide, NHS-biotin, and WGA-HRP, and the resulting data was shown with calculated R and p-values as determined using the simple linear regression software suite in Prism. For the supplementary heatmap output, total area normalized LFQ data found in **Figure 5 - Source**

677 **data 2** was loaded into Morpheus (software.broadinstitute.org/Morpheus) and data points were
678 clustered by the Pearson correlation between all replicates on both columns and rows. This same
679 data was used in Perseus to produce the principle component analysis (PCA). Distinct gene clusters
680 were further analyzed by functional annotation using the DAVID Bioinformatics Resource 6.8.

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Figure 1-Figure supplement 1. Expression, purification, and validation of APEX2 enzyme. His-tagged APEX2 was expressed in BL21(DE3)pLysS cells and purified by a nickel column. 10 μ g of purified enzyme was run out on a 4-12% Bis-Tris gel to confirm purity.

Figure 1–Figure supplement 2. Labeling and efficacy of APEX2 with DNA. (A) APEX2 was first conjugated with DBCO-Maleimide (DBCO-Mal) reagent at 40 equivalents for 5 hours (80% conversion to the singly labeled product). Following desalting, 3 equivalences of Azide-DNA was added to the conjugate and purified by a Nickel column. Both reactions were monitored by LC-MS as shown. (B) 500,000 Expi293 cells were labeled with 0.5 μ M purified APEX2 and DBCO-labeled APEX2 for 2 min. Extent of biotinylation of target cells was quantified by flow cytometry staining with streptavidin-647. (C) The DNA-APEX2 conjugate was shown to be tethered in the presence of the lipidated DNA (purple) and not in the absence (green), as detected by an Anti-His 680 antibody. Unlabeled APEX2 (blue) additionally did not result in a signal shift. Flow cytometry images are representative of one biological replicate.

Figure 1–Figure supplement 3. WGA-HRP pre-incubation time on cells has no effect on labeling efficiency. WGA-HRP was incubated on Expi293 cells for 0-30 min to determine optimal incubation time on ice before labeling. All tested times resulted in similar cell surface biotinylation efficiencies and signified that no incubation time was needed. Flow cytometry images are representative of one biological replicate.

Figure 2–Figure supplement 1. Optimization of APEX2 concentrations on cell by flow cytometry. (A) 500,000 Expi293 cells were labeled for 2 min with increasing amounts of purified APEX2 enzyme and extent of labeling was quantified by flow cytometry staining with streptavidin-647. (B) Varying numbers of Expi293 cells were labeled for 2 min with 0.5 μ M APEX2 to test range of cell numbers for labeling. Flow cytometry images are representative of one biological replicate.

Figure 2-Figure supplement 2. Rank ordered intensities for surface annotated proteins detected in tethered and untethered enzyme samples. (A) A rank ordered list of MS1 intensities (area) for surface annotated proteins found in APEX2 and DNA-APEX2 samples. (B) A rank ordered list of MS1 intensities (area) for surface annotated proteins found in HRP and WGA-HRP samples. The average of two biological replicates are plotted.

Figure 2-Figure supplement 3. Comparison of enrichment for glycosylated and non-glycosylated proteins. Total MS1 intensities (LFQ area) of Uniprot annotated glycoproteins compared to non-glycoproteins in the SURFY database for each labeling method compared to the total area sum intensity.

Figure 2–Figure supplement 4. Target intensities and total plasma membrane protein identifications for DNA-APEX2 and WGA-HRP labeling experiments as a function of time. 500,000 PaTu8902 pancreatic cancer cells were labeled with either 0.5 μ M DNA-APEX2 or 0.5 μ M WGA-HRP for 1 or 2 minutes at 37°C. After cell surface enrichment and mass spectrometry analysis, the plasma membrane derived protein identifications were totaled. Two biological replicates are plotted. **Figure 2–Figure supplement 4–Source data 1** Mass spectrometry analysis results table.

Figure 2-Figure supplement 5. WGA-HRP labeling is N-acetyl-D-glucosamine (GlcNAc) dependent. Biotinylation of RWPE-1 Myc cells with WGA-HRP was determined with (orange) and without (dark blue) 100 mg/mL GlcNAc. There is a significant leftward shift in the degree of labeling in the absence of competing GlcNAc, demonstrating that the enhanced labeling by WGA-HRP is GlcNAc dependent. The degree of labeling is similar to soluble HRP, as shown in light blue. Importantly, presence of GlcNAc in solution did not generally affect HRP labeling as seen by the control in red. The data presented is from one biological replicate.

Figure 2-Figure supplement 6. WGA-HRP can be used to label adherent cells on-plate. Cell surface labeling was compared between labeling adherent cells on a tissue culture plate vs. lifting cells and then performing labeling. Cell surface biotinylation was detected by streptavidin-Alexa Fluor 647. The data presented is from one biological replicate.

Figure 3-Figure supplement 1. Comparison of surface enrichment between replicates for different mass spectrometry methods. (A) The top three methods (NHS-Biotin, Biocytin Hydrazide, and WGA-HRP) were compared for their ability to enrich cell surface proteins on 1.5 M RWPE-1 Control cells by LC-MS/MS after being searched with the Uniprot GOCC Plasma Membrane database. Shown are enrichment levels on the protein, peptide, and average MS1 intensity of top three peptides (LFQ area) levels. (B) The top three methods (NHS-Biotin, Biocytin Hydrazide, and WGA-HRP) were compared for their ability to enrich cell surface proteins on 1.5 M RWPE-1 Control cells by LC-MS/MS after being searched with the entire human Uniprot database. Shown are enrichment levels on the protein, peptide, and average MS1 intensity of top three peptides (LFQ area) levels. Proteins or peptides detected from cell surface annotated proteins (determined by the SURFY database) were divided by the total number of proteins or peptides detected. LFQ areas corresponding to cell surface annotated proteins (SURFY) were divided by the total area sum intensity for each sample. The corresponding percentages for two biological replicates were plotted. **Figure 3-Figure supplement 1-Source data 1** Mass spectrometry analysis results table.

Figure 3–Figure supplement 2. Comparison of replicates for different mass spectrometry methods show that WGA-HRP has comparable reproducibility to NHS-Biotin and Hydrazide labeling. (A) Spearman correlations of total area sum intensity normalized data from replicates of Hydrazide Control and Myc cells. (B) Spearman correlations of total area sum intensity normalized data from replicates of NHS Control and Myc cells. (C) Spearman correlations of total area sum intensity normalized data from replicates of WGA Control and Myc cells.

Figure 4–Figure supplement 1. Venn diagram comparing enriched targets (>2-fold) in Cells and EVs. (A) Surface and secreted targets that were found enriched in the Control EVs (purple) and Control cells (blue) when each is separately compared to Myc EVs and Myc cells, respectively. The 4 overlapping enriched targets in common between Control cells and Control EVs are listed in the center. (B) Surface and secreted targets that were found enriched in the Myc EVs (purple) and Myc cells (blue) when each is separately compared to Control EVs and Control cells, respectively. The 12 overlapping enriched targets in common between Myc cells and Myc EVs are listed in the center.

Figure 5–Figure supplement 1. Heatmap comparison of biological and technical replicates of RWPE-1 Control/Myc cells and EVs. Biological and technical replicates cluster together based on both oncogene status and compartment for EV or cell surface. Proteins with no area values were assigned an imputed value using Perseus. Heatmap clustering is based off of the Pearson correlation between all replicates on both columns and rows. Heatmap was produced using Morpheus, <https://software.broadinstitute.org/Morpheus>. The first number following the sample name denotes the biological replicated and second number denotes the technical replicate.