Supplementary material

Spatial EGFR dynamics and metastatic phenotypes modulated by upregulated EPHB2 and SRC pathways in advanced prostate cancer

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Method S1 | 2D single-particle tracking

Single-particle trajectories were determined from the raw data sets using a three-step process: (i) identifying contiguous regions of pixels; (ii) Gaussian fitting; (iii) building trajectories from coordinates. This approach is similar to those described previously[1-3].

1. Identifying contiguous regions of pixels. A series of 2D images in time trace was processed independently to find FN-IgG-EGFR coordinates. The contiguous regions of pixels, which represent the images of fluorescent particles, were identified on the basis of two criteria: (i) pixels had intensities greater than 3-fold the standard deviation of pixel intensities from areas defined as background (background offset algorithm[4]) and (ii) pixels were above a threshold[4]. Then, a high pass filtering was applied to the image with a 2D Gaussian filter ($\sigma = 5$). The binary image of pixels passing both criteria was later processed by Gaussian fitting.

2. Gaussian fitting. To find out the center of the fluorescent particles, the center of mass of each contiguous region in the binary image was set as the starting point in a Gaussian fitting routine. The highest intensity pixel in a small region around the starting point (5 pixels square) was used as an updated starting. Fits were performed in a square region, of size $\sim 2*\sigma_{psf}$, around the updated starting point. The $\sigma_{psf}$ defines the size of 2D Gaussian approximation to the point spread function. After convergence of the fitting routine (a change in location of fewer than $10^{-5}$ pixels), a normalized cross-correlation was calculated to verify the 2D Gaussian-fitted coordinates. The found coordinates were only considered as positions of FN-IgG-EGFR and used in the further analysis if they exceeded a cross-correlation value of 0.7.

3. Building trajectories from coordinates. The probability of finding a diffusing particle with diffusivity $D$ in two dimensions at a distance greater than $d$ from its starting point after a time $\Delta t$ is given by[5]

$$P(d, \Delta t) = \exp \left[ -\frac{d^2}{4D\Delta t} \right] \quad (1)$$

Trajectories were built from the set of 3D coordinates (x,y, and t) in two steps. First, coordinates identified at time $t$ were compared with coordinates at time $t+\Delta t$ using Eq. 1 where $\Delta t$ is the inverse frame rate of data acquisition. If $P(r, \Delta t)$ was found to be greater than .05, the coordinate at $t+\Delta t$ is associated with the coordinate at $t$ in a trajectory. This process builds short, un-interrupted trajectories. Second, to connect these short trajectories originated from the same targets, the end coordinate of all trajectories is compared with all later starting coordinates of other trajectories using Eq. 1, where $\Delta t$ is now the time interval between the end of the first trajectory and the beginning of the second. The later trajectory with the smallest $\Delta t$ that has a $P(r, \Delta t )> 0.01$ is connected with the first trajectory. This process is continued
until there are no remaining pairs of trajectories that satisfy the criteria. The reconstructed trajectories are further processed into mean-squared displacement to estimate diffusion coefficient.

**Method S2 | Extracting dynamic parameters from MSD**

The typical approach to analyze a single-particle trajectory starts with the calculation of mean-squared displacement (MSD)[6, 7], which describes the average squared distance ($d^2$, $r$ is the position vector) that the particle has explored in space at a given time lag ($\Delta t$):

$$\text{MSD}(\Delta t) = \langle (r(t + \Delta t) - r(t))^2 \rangle$$  \hspace{1cm} (2)

Collected trajectories all started on the apical surfaces of the cells and exhibited an interchange of confined diffusion and Brownian diffusion. The dynamics parameters, the diffusivity ($D$) and the linear dimension of compartments ($L$), are extracted from trajectories by fitting the MSD curves. The $D$ was defined as the linear MSD fitting result of the first 5 MSD points:

$$\text{MSD}(\Delta t) = \langle (r(t + \Delta t) - r(t))^2 \rangle = 4D_{macro}\Delta t$$  \hspace{1cm} (3)

For $L$, the MSD curves were fitted with an equation for confined diffusion[8, 9], and $L$ was defined as the MSD fitting result of the first 10 MSD points:

$$\text{MSD}(\Delta t) \approx \frac{l^2}{3} \left[ 1 - \exp \left( -\frac{\Delta t}{\tau} \right) \right] + 4D_{macro}\Delta t$$  \hspace{1cm} (4)

Confined diffusion is featured by an abrupt change of slope in the MSD curve[10] after a characteristic equilibration time $\tau$.

**Method S3 | 3D single-particle tracking**

TSUNAMI (Tracking of Single particles Using Nonlinear And Multiplexed Illumination) is a feedback-control tracking system which employs a spatiotemporally multiplexed two-photon excitation and temporally demultiplexed detection scheme. Sub-millisecond temporal resolution (under high signal-to-noise conditions) and sub-diffraction tracking precision in all three dimensions have been previously demonstrated[11-13]. Tracking can be performed in a live cell to monitor the movements of fluorescent nanoparticle-tagged EGFRs[13, 14] or ballistically injected fluorescent nanoparticles[15]. The TSUNAMI microscope has been described in detail in our previous study 10. In brief, excitation of 800 nm from a Ti:Al$_2$O$_3$ laser (Mira 900, Coherent) was used for tracking experiments. The optical multiplexer splits the primary laser beam into 4 beams which then passed through a galvo scanning system (6125H, Cambridge Technology). These 4 beams were focused through a 60× N.A. 1.3 silicone oil objective (UPLSAPO 60X, Olympus) to create a tetrahedral excitation profile. The total laser power was ~8mW at the objective back aperture. The photon count rates of a typical ≈40 nm red fluorescent bead (F8770, Thermo Fisher Scientific) were in the range of 200-500 kHz. Signal-to-noise ratios were typically above 20 with 3 kHz background fluorescence signal and 150 Hz background signal from the detector. Temporally demultiplexed detection was performed via time-correlated single-photon counting (TCSPC).
Fluorescence signals were detected by a cooled GaAsP photomultiplier tube (PMT) with 5 mm square active area (H7422PA-40, Hamamatsu) in the non-descanned configuration. The current output from the PMT was amplified through a 2 GHz cutoff bandwidth preamplifier (HFAC-26, Becker and Hickl GmbH) and sent into a photon-counting board (SPC-150, Becker and Hickl GmbH) to be counted and correlated to the 76 MHz reference clock of the laser oscillator. The instrument response function (IRF) was measured to be 230 ps FWHM. Every 5 ms a photon histogram was sampled from the TCSPC module and processed in the software loop run in LabVIEW (National Instruments). The tracking algorithm employs a proportional control to convert the error signals to new stage positions. New voltages were sent out through a DAQ card (PCle-6353, National Instruments) to their respective actuators – galvo mirrors for x and y tracking and piezo objective stage for z tracking. The saved voltages were converted to build a 3D tracking trajectory.

**Method S4 | Two-photon laser scanning microscope (2P-LSM)**

The excitation light for laser scanning imaging shared the same path as the non-delayed beam in the multiplexer, the other 3 beams were blocked with shutters during laser scanning. By the nature of 2P excitation, excitation of 800 nm from a Ti:Al₂O₃ laser (Mira 900, Coherent) is able to excite Hochest 33258 (Hochest 33258, H3569, Thermo Fisher Scientific), red fluorescence nanoparticle (F8770, Thermo Fisher Scientific), and Cellmask™ Deep Red (C10046, Thermo Fisher Scientific). The emission of these three fluorophores was collected simultaneously with three individual photomultiplier tubes (PMT).

The excitation light for laser scanning imaging shared the same path as the non-delayed beam in the multiplexer. The emission light was collected through the same objective and transmitted through the dichroic filter to be condensed onto three PMTs. The emission light was split into three color bands by two dichroic mirrors before being condensed onto each PMT (H7422PA-40, Hamamatsu). The first dichroic mirror picks of the blue-green spectrum with a reflection edge at 550 nm (FF552-Di02-25x36, Semrock) the second dichroic splits the remaining light into orange and red components with an edge at 640 nm (FF640-FDi01-25x36, Semrock). Each PMT has a bandpass filter specific to the fluorescent targets used for the experiment, 460-500nm for nuclear stain (Hochest 33258, H3569, Thermo Fisher Scientific), 570-610nm for red fluorescent nanoparticle (F8770, Thermo Fisher Scientific) and 680-720nm for membrane stain (Cellmask™ Deep Red, C10046, Thermo Fisher Scientific). The mirror scanning and data acquisition were handled by a single DAQ card (PCle-6353, National Instruments) with a custom code interface in LabVIEW. Pixel dwell times were 2.6 microseconds which correspond to a frame rate of approximately 1.4 Hz. Image sizes were typically 51 × 51 µm².
Method S5 | Data processing for 3D single-particle tracking and 2P scanning imaging

All data processing was performed in MATLAB (Mathworks). Saved in a binary format, the trajectory raw data contained photon counts and voltage outputs from the actuators (i.e. the xy scanning galvo mirrors (6125H, Cambridge Technology) and the objective z-piezo stage (P-726 PIFOC, PI)) at each 5 ms time point. Trajectories were plotted by simply connecting particle positions of consecutive time points. 2P-LSM raw images were read into MATLAB from binary files and denoised with a median filter before a 1D interpolation along the z dimension. To segment the cellular compartments, we used a simple intensity threshold technique that converts the image to a binary. Thresholds were selected at each z plane to account for variation in noise and brightness through the z-stack. The binary images were used as a mask to plot the cell isocontour. Because trajectories were measured with the same analog output device as the 2P-LSM images they can be directly overlaid with the cell compartment isocontour with no conversion or scaling required.

Method S6 | 3D Inward movement analysis

The 3D inward movement analysis is adapted from Picco’s 2D inward movement analysis[16], and it has been used to analyze the anti-PDL1 antibody-induced PD-1 internalization[17]. The 3D inward movement analysis is a two-step procedure based on the 2P scanning imaging and 3D single-particle tracking. Firstly, the 3D-stacked live-cell image was acquired by 2P-LSM. Secondly, the fluorescently labeled EGFR was tracked by the TSUNAMI microscope. Then the EGFR trajectory was coregistered with the 3D cell image. The distance and velocity of the inward movement were then derived from the collected trajectories.

The 3D inward movement analysis is a two-step procedure based on the 2P scanning imaging and 3D single-particle tracking. Firstly, the 3D-stacked live-cell image was acquired by 2P-LSM. Secondly, the fluorescently labeled EGFR was tracked by the TSUNAMI microscope. Then the EGFR trajectory was coregistered with the 3D cell image. The distance and velocity of the inward movement were then derived from the collected trajectories. To visualize the plasma membrane and nuclei, cells were stained with CellMask™ Deep Red and Hoechst 33258. To define the orientation of inward movement, a tangent plane on the plasma membrane was derived using the start point of a trajectory and the reconstructed 3D isocontour of the plasma membrane. The normal vector which is perpendicular to the tangent plane is the axis of inward movement. Then the trajectory was projected to the normal vector, and the displacement along the normal vector is defined as the inward movement. For sample preparation, cells were seeded onto optical imaging 8-well chambered coverglass (155409, Thermo Fisher Scientific) with the cell density of 1×10^5 cells per well and allowed to grow to ~70% cell confluency. Before tracking experiments, cells were stained with a mixture of Hoechst 33258 (H3569, Thermo Fisher Scientific,
1:1000 dilution in complete medium) and CellMask™ Deep Red (C10046, Thermo Fisher Scientific, 1:1000 dilution in complete medium) for 10 minutes at 37°C. After membrane staining, the staining buffer was replaced with the antibody solution (antibody-conjugated fluorescent nanoparticles at 100 pM). The reaction was incubated for 10 minutes at 37°C and the antibody solution was removed, subsequently. Sample cells were washed twice using PBS to remove the unbound fluorescent nanoparticles. Upon completion of membrane staining and antibody labeling, the chambered coverglass was immediately placed on the TSUNAMI microscope for tracking experiments. Two to four trajectories (duration ranged from 1-10 minutes) were typically obtained from each well after the EGF stimulation (20 ng/mL EGF (recombinant human epidermal growth factor, PHG0311L, Thermo Fisher Scientific). The volumes of all solutions and washing buffers used in staining were 200 µl per well. All data processing was performed in MATLAB (Mathworks) and saved in a binary format. The trajectory raw data contained photon counts and voltage outputs from the actuators (i.e. the xy scanning galvo mirrors (6125H, Cambridge Technology) and the objective z-piezo stage (P-726 PIFOC, PI)) at each 5 ms time point. Trajectories were plotted by simply connecting particle positions of consecutive time points.
A panel of five prostate cell lines was evaluated with the TReD assay to characterize their EGFR diffusivity ($D$) and compartment size ($L$). There is a trend of increased EGFR diffusivities and compartment sizes associated with advanced malignancy. PC3 exhibited significantly the highest diffusivity and largest compartment size. The number of trajectories collected from each cell line is shown on each bar. Statistical comparison was performed using unpaired t-test, where the asterisk represents statistical significance: **p<0.01, *p<0.05. The error bar represents the standard error of the mean.
Figure S2 | Evaluation of EGFR internalization by a 3D single-particle tracking technique

(A) The schematic explains that the direction of inward movement is defined as the normal vector of the tangent plane on the plasma membrane at the start point of a trajectory. Then the trajectory was projected on the normal vector to derive the inward movement. **(B)** A representative trajectory demonstrates the internalization of an EGFR in an A431 cell whose plasma membrane (PM) and nucleus were stained with CellMask Deep Red and Hoechst, respective. The inward movement of the trajectory is plotted in **(C)**. **(D)** A 2P-scanning image of LNCaP cell and the inward movement of the EGFR into the cell. **(E), (F)** EGFR internalization was characterized by the trajectory-derived parameters: inward movement and velocity. The error bar represents the standard error of the mean from 9-20 measurements. All statistical analysis was performed using the unpaired t-test. The asterisk represents the level of statistical significance for t-test: *** $p < 0.001$. 
Figure S3 | Process of quantification of cortical actin on the apical side of the plasma membrane

The quantification of the fluorescence intensity of cortical actin was conducted using the software: ZEN 2.3 lite. The xz and yz projections of 2 µm width on the xy plan were extracted from the Z-stack of SR-SIM images. The fluorescence intensity of actin along the apical side of the plasma membrane and the brightest region were measured. To make a fair comparison among batches of images, the measured intensities were normalized with the average intensity of brightest region in the same projection. The average of the normalized fluorescence intensity of actin was taken as an indicator of the density of cortical actin. The procedure of image-based quantification of cortical actin was summarized below, and more representative fluorescence profiles were presented in Figure S4 and Figure S10.
Figure S4 | Quantification of cortical actin on the apical side of the plasma membrane in Lat-B treated cells

(A) The representative images of xz projections of cells with fluorescently labeled actin filaments (white) and nuclei (blue), which are also shown in Figure 2. The corresponding profiles of the normalized fluorescence intensity along the apical side of the plasma membrane indicated by yellow dashed lines. The fluorescence intensities are normalized and presented as an arbitrary unit (a.u.). (B) Quantification of cortical actin-based on the measured fluorescence intensities. The averages of normalized intensities were presented, and the number of projections analyzed is labeled on each bar. All statistical analysis was performed using the unpaired t-test. The asterisk represents the level of statistical significance for t-test: * $p < 0.05$, *** $p < 0.001$. The error bar represents the standard error of the mean.
Figure S5 | Unsupervised hierarchical clustering of DE gene expression in EPHB-mediated forward signaling.

(A) Unsupervised hierarchical clustering of differentially expressed (DE) genes (171) in Src signaling. LNCaP (LN), LNCaP-Abl (ABL) and PC3 are distinct clusters separated from the other clusters. (B) Unsupervised hierarchical clustering of DE gene expression in Eph-ephrin signaling. The dashed white lines delineate the gene sets upregulated in LNCaP-Abl cells. (C) ScRNA-seq showed that the majority of genes in the EPHB-mediated forward pathway are upregulated in PC3 cells. LNCaP and LNCaP-Abl cells display heterogeneity but are clustered together based on DE genes in EPH-Epherin and EPHB-mediated signaling.
Figure S6 | The transcript expression of actin-binding protein genes downstream to Rho GTPases and their associated effectors in PC cells.

The transcript expression of genes in actin-binding proteins and regulators are based on scRNA-seq data and shown in RPKM. Most of the gene transcripts are upregulated in more aggressive PC3 cells. The transcript expression of genes in actin-binding proteins and regulators are based on scRNA-seq data and shown in RPKM.
Figure S7 | Eph-ephrin and Src signaling expression profile in prostate circulating tumor cells.

(A) t-Distributed Stochastic Neighbor Embedding (t-SNE) scatter plot of nine prostate cancer patients CTCs based on SRC and EphB/ephrinB signaling expression profile. (B) Four different clusters were identified and projected in t-SNE scatter plot. (C) Proportion of the CTCs in each cluster per patient.
Figure S8 | Upregulation of DE genes in Src and EphB2 pathways predicts high grades and poor disease-free survival in TCGA prostate cancer patient cohort. 

(A) Expression pattern of 21 genes differentiates high-grade tumors with Gleason score 9-10 from tumors with Gleason score ≤ 8. (B) The alternation of four additional genes predicts poor prognosis using Kaplan-Meier estimation analysis.
Figure S9 | Dasatinib attenuated cell motility, invasion and EGFR diffusion in PC3.

(A)-(C) The attenuation of proliferation, migration, invasion of three PC cells (LNCaP, DU145, and PC3) treated with dasatinib, an inhibitor to EPHB/SRC pathways. The image-based IncuCyte assays allow us to conduct the time-lapse analysis of cell proliferation, migration, and invasion of the cells treated with or without dasatinib. The dasatinib significantly inhibits the proliferation, migration, and invasion of the highly metastatic prostate cancer cells (PC3). The dasatinib also significantly decreases the EGFR diffusivity in PC3 cells. The mean value of each bar was measured at the end time of each assay or at 48th-hour. All statistical analysis was performed using the unpaired t-test. The asterisk represents the level of statistical significance for t-test: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. The error bar represents the standard error of the mean. The RWD stands for relative wound density.
Figure S10 | Quantification of cortical actin on the apical side of the plasma membrane in gene-knocked down cells.

(A) The representative images of xz projections of cells with fluorescently labeled actin filaments (white) and nuclei (blue), which are also shown in Figure 5c. These plots demonstrate the corresponding profiles of the normalized fluorescence intensity along the apical side of the plasma membrane. (B) Quantification of cortical actin-based on the measured fluorescence intensities. The fluorescence intensities are normalized and presented as an arbitrary unit (a.u.). The number of projections analyzed is labeled on each bar. All statistical analysis was performed using the unpaired t-test. The asterisk represents the level of statistical significance for t-test: * $p < 0.05$, *** $p < 0.001$. The error bar represents the standard error of the mean.
Figure S11 | Knockdowns of SRC and EPHB2 mRNA affect migration and invasion.

(A), (B) The imaging-based assays allow us to conduct the time-lapse analysis of cell migration and invasion of the siRNA-treated cells. The error bar represents the standard deviation. All statistical analysis was performed using the unpaired t-test. The asterisk represents the level of statistical significance for t-test: ** $p < 0.01$, * $p < 0.05$. 
Figure S12 | EGFR expression of prostate cancer cell lines

(A) EGFR expression and phosphorylation of prostate cancer cell lines. (B) Immunocytochemistry images of cells fluorescently labeled with Anti-EGFR Affibody® Molecule FITC, the Alexa Fluor™ 633 Phalloidin, and the Hoechst 33258.
Figure S13 | Western blots

Western blot evaluation for (A) phosphorylated EGFR, EPHB2, SRC and (B) total EGFR. The duplicate experiments are shown side by side. The specific bands for those proteins are indicated by blue arrows. The molecular weight markers are labeled on the right sides.
# Table S1 | Differentially expressed genes of EPH, EPHB forward, and SRC pathways in PC3.

## EPH-Ephrin signaling

| Gene Name | Up (+) or down (-) regulation in PC3 | entrez-gene ID | entrez-gene name |
|-----------|-------------------------------------|----------------|------------------|
| ARPC2#    | +                                   | 10109          | ARPC2 : actin related protein 2/3 complex, subunit 2, 34kDa |
| EPHB2*    | +                                   | 2048           | EPHB2 : EPH receptor B2 |
| MYL9#     | +                                   | 10398          | MYL9 : myosin, light chain 9, regulatory |
| MYL12A*   | +                                   | 10627          | MYL12A : myosin, light chain 12A, regulatory, non-sarcomeric |
| FYN*#     | +                                   | 2534           | FYN : FYN proto-oncogene, Src family tyrosine kinase |
| EFNB1*#   | +                                   | 1947           | EFN1B : ephrin-B1 |
| PTK2*     | +                                   | 5747           | PTK2 : protein tyrosine kinase 2 |
| EFNB3*#   | +                                   | 1949           | EFN3 : ephrin-B3 |
| EPHA2*#   | +                                   | 1969           | EPHA2 : EPH receptor A2 |
| MYL6      | +                                   | 4637           | MYL6 : myosin, light chain 6, alkali, smooth muscle and non-muscle |
| RAC1*     | +                                   | 5879           | RAC1 : ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) |
| CFL1*     | +                                   | 1072           | CFL1 : cofilin 1 (non-muscle) |
| ROCK2*    | +                                   | 9475           | ROCK2 : Rho-associated, coiled-coil containing protein kinase 2 |
| SHB*      | +                                   | 6461           | SHB : Src homology 2 domain containing adaptor protein B |
| TIAM1*    | +                                   | 7074           | TIAM1 : T-cell lymphoma invasion and metastasis 1 |
| ARHGECF28 | +                                   | 64283          | ARHGECF28 : Rho guanine nucleotide exchange factor (GEF) 28 |
| ARPC1A    | +                                   | 10552          | ARPC1A : actin related protein 2/3 complex, subunit 1A, 41kDa |
| EPHA1*    | -                                   | 2041           | EPHA1 : EPH receptor A1 |
| MYH14     | -                                   | 79784          | MYH14 : myosin, heavy chain 14, non-muscle |
| MYH10     | -                                   | 4628           | MYH10 : myosin, heavy chain 10, non-muscle |

## EPHB forward signaling

| Gene Name | Up (+) or down (-) regulation in PC3 | entrez-gene ID | entrez-gene name |
|-----------|-------------------------------------|----------------|------------------|
| EPHB2**   | +                                   | 2048           | EPHB2 : EPH receptor B2 |
| MAPK3     | +                                   | 5595           | MAPK3 : mitogen-activated protein kinase 3 |
| PTK2**    | +                                   | 5747           | PTK2 : protein tyrosine kinase 2 |
| PXN       | +                                   | 5829           | PXN : paxillin |
| Gene Name   | Up (+) or Down (-) regulation in PC3 | entrez-gene ID | entrez-gene name |
|-------------|--------------------------------------|----------------|-----------------|
| RAC1**      | +                                    | 5879           | RAC1 : ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) |
| RAP1B       | +                                    | 5908           | RAP1B : RAP1B, member of RAS oncogene family |
| GRB2        | +                                    | 2885           | GRB2 : growth factor receptor-bound protein 2 |
| EFNB3**     | +                                    | 1949           | EFNB3 : ephrin-B3 |
| EFNB1**     | +                                    | 1947           | EFNB1 : ephrin-B1 |
| MAP4K4      | +                                    | 9448           | MAP4K4 : mitogen-activated protein kinase kinase kinase 4 |
| PIK3R1      | -                                    | 5295           | PIK3R1 : phosphoinositide-3-kinase, regulatory subunit 1 (alpha) |
| KRAS        | -                                    | 3845           | KRAS : Kirsten rat sarcoma viral oncogene homolog |
| SRC signaling |                           |                |                  |
| Gene Name   | Up (+) or Down (-) regulation in PC3 | entrez-gene ID | entrez-gene name |
| EPHB2       | +                                    | 2048           | EPHB2 : EPH receptor B2 |
| SPRY2       | +                                    | 10253          | SPRY2 : sprouty RTK signaling antagonist 2 |
| EYA4        | +                                    | 2070           | EYA4 : EYA transcriptional coactivator and phosphatase 4 |
| ETS1        | +                                    | 2113           | ETS1 : v-ets avian erythroblastosis virus E26 oncogene homolog 1 |
| ETS2        | +                                    | 2114           | ETS2 : v-ets avian erythroblastosis virus E26 oncogene homolog 2 |
| ACTN1       | +                                    | 87             | ACTN1 : actinin, alpha 1 |
| RPS27A      | +                                    | 6233           | RPS27A : ribosomal protein S27a |
| RIN3        | +                                    | 79890          | RIN3 : Ras and Rab interactor 3 |
| MET         | +                                    | 4233           | MET : MET proto-oncogene, receptor tyrosine kinase |
| FGF1        | +                                    | 2246           | FGF1 : fibroblast growth factor 1 (acidic) |
| PALM2-AKAP2 | +                                    | 445815         | PALM2-AKAP2 : PALM2-AKAP2 readthrough |
| FGFR1       | +                                    | 2260           | FGFR1 : fibroblast growth factor receptor 1 |
| SDC4        | +                                    | 6385           | SDC4 : syndecan 4 |
| FLNA        | +                                    | 2316           | FLNA : filamin A, alpha |
| ANXA1       | +                                    | 301            | ANXA1 : annexin A1 |
| ANXA2       | +                                    | 302            | ANXA2 : annexin A2 |
| ANXA7       | +                                    | 310            | ANXA7 : annexin A7 |
| CNTNAP1     | +                                    | 8506           | CNTNAP1 : contactin associated protein 1 |
| ABLIM3      | +                                    | 22885          | ABLIM3 : actin binding LIM protein family, member 3 |
| ARAF        | +                                    | 369            | ARAF : A-Raf proto-oncogene, serine/threonine kinase |
| MST1R       | +                                    | 4486           | MST1R : macrophage stimulating 1 receptor |
| ARHGDIA     | +                                    | 396            | ARHGDIA : Rho GDP dissociation inhibitor (GDI) alpha |
| ARHGDIB     | +                                    | 397            | ARHGDIB : Rho GDP dissociation inhibitor (GDI) beta |
| WNK4        | +                                    | 65266          | WNK4 : WNK lysine deficient protein kinase 4 |
| Gene      | +   | Number | Description                                                                 |
|-----------|-----|--------|-----------------------------------------------------------------------------|
| FYN       | +   | 2534   | FYN : FYN proto-oncogene, Src family tyrosine kinase                        |
| ZYX       | +   | 7791   | ZYX : zyxin                                                                 |
| AXL       | +   | 558    | AXL : AXL receptor tyrosine kinase                                          |
| SPTBN1    | +   | 6711   | SPTBN1 : spectrin, beta, non-erythrocytic 1                                 |
| TFG       | +   | 10342  | TFG : TRK-fused gene                                                         |
| TNFRSF11A | +   | 8792   | TNFRSF11A : tumor necrosis factor receptor superfamily, member 11a, NFKB activator |
| F2R       | +   | 2149   | F2R : coagulation factor II (thrombin) receptor                             |
| EPB41L3   | +   | 23136  | EPB41L3 : erythrocyte membrane protein band 4.1-like 3                      |
| IQGAP1    | +   | 8826   | IQGAP1 : IQ motif containing GTPase activating protein 1                     |
| NEDD4     | +   | 4734   | NEDD4 : neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase |
| GNAI2     | +   | 2771   | GNAI2 : guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2 |
| NMT1      | +   | 4836   | NMT1 : N-myristoyltransferase 1                                              |
| KRT19     | +   | 3880   | KRT19 : keratin 19, type I                                                  |
| PTTG1IP   | +   | 754    | PTTG1IP : pituitary tumor-transforming 1 interacting protein                |
| GPC1      | +   | 2817   | GPC1 : glypican 1                                                           |
| TNS3      | +   | 64759  | TNS3 : tensin 3                                                             |
| STAT6     | +   | 6778   | STAT6 : signal transducer and activator of transcription 6, interleukin-4 induced |
| MMP14     | +   | 4323   | MMP14 : matrix metallopeptidase 14 (membrane-inserted)                       |
| CAPG      | +   | 822    | CAPG : capping protein (actin filament), gelsolin-like                       |
| CAPN2     | +   | 824    | CAPN2 : calpain 2, (m/II) large subunit                                     |
| GRB2      | +   | 2885   | GRB2 : growth factor receptor-bound protein 2                               |
| CAV1      | +   | 857    | CAV1 : caveolin 1, caveolae protein, 22kDa                                  |
| NR3C1     | +   | 2908   | NR3C1 : nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) |
| TGFBR2    | +   | 7048   | TGFBR2 : transforming growth factor, beta receptor II (70/80kDa)            |
| TGM2      | +   | 7052   | TGM2 : transglutaminase 2                                                   |
| AFAP1     | +   | 60312  | AFAP1 : actin filament associated protein 1                                 |
| PVRL3     | +   | 25945  | PVRL3 : poliovirus receptor-related 3                                       |
| TIAM1     | +   | 7074   | TIAM1 : T-cell lymphoma invasion and metastasis 1                            |
| LAMC2     | +   | 3918   | LAMC2 : laminin, gamma 2                                                    |
| P2RY2     | +   | 5029   | P2RY2 : purinergic receptor P2Y, G-protein coupled, 2                        |
| KITLG     | +   | 4254   | KITLG : KIT ligand                                                          |
| CD44      | +   | 960    | CD44 : CD44 molecule (Indian blood group)                                   |
| CD47      | +   | 961    | CD47 : CD47 molecule                                                        |
| H3F3B     | +   | 3021   | H3F3B : H3 histone, family 3B (H3.3B)                                       |
| AKAP2     | +   | 11217  | AKAP2 : A kinase (PRKA) anchor protein 2                                     |
| CDH2      | +   | 1000   | CDH2 : cadherin 2, type 1, N-cadherin (neuronal)                             |
| HIF1A     | +   | 3091   | HIF1A : hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) |
| Gene Symbol | Gene Number | Protein Description |
|-------------|-------------|---------------------|
| CAV2        | + 858       | CAV2 : caveolin 2    |
| TRIP6       | + 7205      | TRIP6 : thyroid hormone receptor interactor 6 |
| CFL1        | + 1072      | CFL1 : cofilin 1 (non-muscle) |
| GNG2        | + 54331     | GNG2 : guanine nucleotide binding protein (G protein), gamma 2 |
| PFN1        | + 5216      | PFN1 : profilin 1    |
| TRIP10      | + 9322      | TRIP10 : thyroid hormone receptor interactor 10 |
| CHRN1B1     | + 1140      | CHRN1B1 : cholinergic receptor, nicotinic, beta 1 (muscle) |
| TXK         | + 7294      | TXK : TXK tyrosine kinase |
| PRKD3       | + 23683     | PRKD3 : protein kinase D3 |
| CNN3        | + 1266      | CNN3 : calponin 3, acidic |
| PLA2G4A     | + 5321      | PLA2G4A : phospholipase A2, group IVA (cytosolic, calcium-dependent) |
| PLAUR       | + 5329      | PLAUR : plasminogen activator, urokinase receptor |
| PLAU        | + 5328      | PLAU : plasminogen activator, urokinase |
| HSPA2       | + 3306      | HSPA2 : heat shock 70kDa protein 2 |
| PLSCR1      | + 5359      | PLSCR1 : phospholipid scramblase 1 |
| ARHGEF6     | + 9459      | ARHGEF6 : Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6 |
| VCL         | + 7414      | VCL : vinculin       |
| ROCK2       | + 9475      | ROCK2 : Rho-associated, coiled-coil containing protein kinase 2 |
| EZR         | + 7430      | EZR : ezrin          |
| SHB         | + 6461      | SHB : Src homology 2 domain containing adaptor protein B |
| TNC         | + 3371      | TNC : tenascin C     |
| POLR2C      | + 5432      | POLR2C : polymerase (RNA) II (DNA directed) polypeptide C, 33kDa |
| POLR2L      | + 5441      | POLR2L : polymerase (RNA) II (DNA directed) polypeptide L, 7.6kDa |
| CDCP1       | + 64866     | CDCP1 : CUB domain containing protein 1 |
| DAB2        | + 1601      | DAB2 : Dab, mitogen-responsive phosphoprotein, homolog 2 (Drosophila) |
| PPP2R5E     | + 5529      | PPP2R5E : protein phosphatase 2, regulatory subunit B, epsilon isoform |
| CNN2        | + 1265      | CNN2 : calponin 2    |
| SH3GLB1     | + 51100     | SH3GLB1 : SH3-domain GRB2-like endophilin B1 |
| EFNB3       | + 1949      | EFNB3 : ephrin-B3    |
| PRKCA       | + 5578      | PRKCA : protein kinase C, alpha |
| ARHGEF4     | + 50649     | ARHGEF4 : Rho guanine nucleotide exchange factor (GEF) 4 |
| MAPK3       | + 5595      | MAPK3 : mitogen-activated protein kinase 3 |
| IL6ST       | + 3572      | IL6ST : interleukin 6 signal transducer |
| GJA1        | + 2697      | GJA1 : gap junction protein, alpha 1, 43kDa |
| PHF19       | + 26147     | PHF19 : PHD finger protein 19 |
| Gene   | ID     | Description                                                                 |
|--------|--------|------------------------------------------------------------------------------|
| PAK6   | 56924  | PAK6 : p21 protein (Cdc42/Rac)-activated kinase 6                            |
| SIRPA  | 140885 | SIRPA : signal-regulatory protein alpha                                       |
| ITGA2B | 3674   | ITGA2B : integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIa complex, antigen CD41) |
| HGS    | 9146   | HGS : hepatocyte growth factor-regulated tyrosine kinase substrate           |
| ITGB1  | 3688   | ITGB1 : integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) |
| PTK2   | 5747   | PTK2 : protein tyrosine kinase 2                                             |
| GAB2   | 9846   | GAB2 : GRB2-associated binding protein 2                                      |
| ASAP1  | 50807  | ASAP1 : ArfGAP with SH3 domain, ankyrin repeat and PH domain 1               |
| CCDC88A| 55704  | CCDC88A : coiled-coil domain containing 88A                                  |
| CD59   | 966    | CD59 : CD59 molecule, complement regulatory protein                           |
| CD63   | 967    | CD63 : CD63 molecule                                                         |
| MYL12A | 10627  | MYL12A : myosin, light chain 12A, regulatory, non-sarcomeric                 |
| PXN    | 5829   | PXN : paxillin                                                               |
| RAC1   | 5879   | RAC1 : ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) |
| SGK1   | 6446   | SGK1 : serum/glucocorticoid regulated kinase 1                                |
| HBEGF  | 1839   | HBEGF : heparin-binding EGF-like growth factor                               |
| DUSP4  | 1846   | DUSP4 : dual specificity phosphatase 4                                       |
| DUSP5  | 1847   | DUSP5 : dual specificity phosphatase 5                                       |
| DLGAP4 | 22839  | DLGAP4 : discs, large (Drosophila) homolog-associated protein 4              |
| LAMA5  | 3911   | LAMA5 : laminin, alpha 5                                                     |
| LAMB1  | 3912   | LAMB1 : laminin, beta 1                                                      |
| LAMB3  | 3914   | LAMB3 : laminin, beta 3                                                      |
| SH3KBP1| 30011  | SH3KBP1 : SH3-domain kinase binding protein 1                                |
| ADAM12 | 8038   | ADAM12 : ADAM metallopeptidase domain 12                                     |
| S1PR3  | 1903   | S1PR3 : sphingosine-1-phosphate receptor 3                                   |
| PTP4A2 | 8073   | PTP4A2 : protein tyrosine phosphatase type IVA, member 2                      |
| EFN1   | 1947   | EFN1 : ephrin-B1                                                             |
| EPHA2  | 1969   | EPHA2 : EPH receptor A2                                                       |
| SPRY4  | 81848  | SPRY4 : sprouty RTK signaling antagonist 4                                   |
| PTPRK  | 5796   | PTPRK : protein tyrosine phosphatase, receptor type, K                       |
| ENO1   | 2023   | ENO1 : enolase 1, (alpha)                                                    |
| FSD1   | 79187  | FSD1 : fibronectin type III and SPRY domain containing 1                     |
| SMAD3  | 4088   | SMAD3 : SMAD family member 3                                                  |
| ERBB3  | 2065   | ERBB3 : erb-b2 receptor tyrosine kinase 3                                    |
| EFS    | 10278  | EFS : embryonal Fyn-associated substrate                                     |
| Symbol  | Gene ID  | Description                                                                 |
|---------|----------|-----------------------------------------------------------------------------|
| SHROOM2 | 357      | SHROOM2: shroom family member 2                                              |
| PTK2B   | 2185     | PTK2B: protein tyrosine kinase 2 beta                                       |
| OCLN    | 100506658| OCLN: occludin                                                              |
| PIK3R3  | 8503     | PIK3R3: phosphoinositide-3-kinase, regulatory subunit 3 (gamma)             |
| CD2AP   | 23607    | CD2AP: CD2-associated protein                                               |
| KLK3    | 354      | KLK3: kallikrein-related peptidase 3                                        |
| AR      | 367      | AR: androgen receptor                                                       |
| IGF1    | 3479     | IGF1: insulin-like growth factor 1 (somatomedin C)                         |
| SLC9A2  | 6549     | SLC9A2: solute carrier family 9, subfamily A (NHE2, cation proton antiporter 2), member 2 |
| CENPV   | 201161   | CENPV: centromere protein V                                                 |
| FRMPD2  | 143162   | FRMPD2: FERM and PDZ domain containing 2                                   |
| STAT3   | 6774     | STAT3: signal transducer and activator of transcription 3 (acute-phase response factor) |
| STAT5B  | 6777     | STAT5B: signal transducer and activator of transcription 5B                |
| EPM2A   | 7957     | EPM2A: epilepsy, progressive myoclonus type 2A, Lafora disease (laforin)   |
| FUBP1   | 8880     | FUBP1: far upstream element (FUSE) binding protein 1                        |
| GRIK2   | 2898     | GRIK2: glutamate receptor, ionotopic, kainate 2                            |
| THRB    | 7066     | THRB: thyroid hormone receptor, beta                                        |
| NANS    | 54187    | NANS: N-acetylneuraminic acid synthase                                      |
| CDH1    | 999      | CDH1: cadherin 1, type 1                                                   |
| GNB2L1  | 10399    | GNB2L1: guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 |
| CDKN1B  | 1027     | CDKN1B: cyclin-dependent kinase inhibitor 1B (p27, Kip1)                    |
| TUB     | 7275     | TUB: tubby bipartite transcription factor                                   |
| PIK3R1  | 5295     | PIK3R1: phosphoinositide-3-kinase, regulatory subunit 1 (alpha)            |
| TRPV6   | 55503    | TRPV6: transient receptor potential cation channel, subfamily V, member 6 |
| SYTL2   | 54843    | SYTL2: synaptotagmin-like 2                                                 |
| UTRN    | 7402     | UTRN: utrophin                                                             |
| PRKACB  | 5567     | PRKACB: protein kinase, cAMP-dependent, catalytic, beta                     |
| PRKAR2B | 5577     | PRKAR2B: protein kinase, cAMP-dependent, regulatory, type II, beta          |
| PRKD1   | 5587     | PRKD1: protein kinase D1                                                    |
| CTNNA1  | 1495     | CTNNA1: catenin (cadherin-associated protein), alpha 1, 102kDa             |
| PTEN    | 5728     | PTEN: phosphatase and tensin homolog                                        |
| PTP4A1  | 7803     | PTP4A1: protein tyrosine phosphatase type IVA, member 1                      |
| JUP   | -   | 3728  | JUP : junction plakoglobin |
|-------|-----|-------|----------------------------|
| STX17 | -   | 55014 | STX17 : syntaxin 17        |
| KLK2  | -   | 3817  | KLK2 : kallikrein-related peptidase 2 |
| KRAS  | -   | 3845  | KRAS : Kirsten rat sarcoma viral oncogene homolog |
| RAP1B | -   | 5908  | RAP1B : RAP1B, member of RAS oncogene family |
| DUSP2 | -   | 1844  | DUSP2 : dual specificity phosphatase 2 |
| LEPR  | -   | 3953  | LEPR : leptin receptor     |
| EPHA1 | -   | 2041  | EPHA1 : EPH receptor A1    |
Table S2 | The qRT-PCR primer sequences for validation of DE gene expression

| Primer Name  | Sequence (5'-3')               | Product Length |
|--------------|--------------------------------|----------------|
| ARHGEF28_F   | GTACGTTGACATCTCCCCCG           | 115            |
| ARHGEF28_R   | TGAGGGCGGTGGAATTGA             | 115            |
| ARPC1A_F     | GACCGCATTGTCACCCGG            | 114            |
| ARPC1A_R     | CACAAAGTAGCTGCGCAT            | 114            |
| ARPC2_F      | CTGTTCCCTTCCGACACCAA          | 141            |
| ARPC2_R      | GAAGTCAGACGTTTTCGCCC          | 141            |
| AXIN2_F      | GCTAGGAGTGGGTCATGTA           | 121            |
| AXIN2_R      | TGTGTCAATGGGTAGGGCACC         | 121            |
| CTNNB1_F     | GGAGGAAGGTCAGGAGGACGA         | 148            |
| CTNNB1_R     | ATGGTCAACGCGTTATTTTCAA        | 148            |
| EFNA1_F      | AAGACGCTGCTGTTGAGTTGA         | 139            |
| EFNA1_R      | GCAGCTGAGCAGTGCTATGTA         | 139            |
| EFNB1_F      | GCTTATCCTGGGAAATGGG           | 126            |
| EFNB1_R      | TACCGTGAAGTGGAGGGGA           | 126            |
| EFNB3_F      | GGACGAAGATGCGCCCTGGGAA        | 131            |
| EFNB3_R      | CCCCAAGACTAAGCCACTTG          | 131            |
| EPHA1_2_F    | AATCAGGGTGCAAGGAGCA           | 91             |
| EPHA1_2_R    | ACCGATAGCCTGACTGTCGCA         | 91             |
| EPHA1_F      | GTGAGGTGGTTTCCAGTGTCA         | 140            |
| EPHA1_R      | TTGGCTAAGGTTCAAGGCCCA         | 140            |
| EPHB2_F      | AGCCATAAAAAGCCCACCA          | 150            |
| EPHB2_R      | TGCTACTCGCCCTCTGCAT           | 150            |
| FYN_R        | ATACTTCCCCAAAACGTGCCATT       | 135            |
| FZD1_F       | CACCAACACGCAAACAGGGGG         | 118            |
| FZD1_R       | AGGATTGGGACGAACTCCAC          | 118            |
| FZD2_F       | CTGGAGACCACCTCCTTCACT         | 74             |
| FZD2_R       | CGCTCGCCAGAAACTGTGA           | 74             |
| ID1_F        | GGCAGCATTGCCGTCCT            | 101            |
| ID1_R        | ACGTACATTCCCTTGCCTCC          | 101            |
| LATS2_F      | CTGACTTGCTGGGTGGG             | 114            |
| LATS2_R      | CCTCGGGTGCGTAGTGAGTTT         | 114            |
| MYH14_F      | CAGCAGCGGTGTTGATGTA           | 128            |
| MYH14_R      | TGATGCAGTGAATTGCGG            | 128            |
| MYL12A_F     | CAACACAGGACTATACCA            | 135            |
| MYL12A_R     | GAGAGGCCCTCTGTGGAACCTGTA      | 135            |
| PTK2_F       | TCCCCAGAGCTCCTCAGGAAT         | 149            |
| Primer Name  | Sequence (5'-3')               | Product Length |
|-------------|--------------------------------|----------------|
| RAC1_F      | TGCATTGTGTGGCCGAGAAC           | 102            |
| RAC1_R      | GAGAGCAAGTGTCTGACCT            | 102            |
| ROCK2_F     | ACGTGGAAGAAGTTGCTGGATG         | 146            |
| ROCK2_R     | TTCTGCCCTTCTACTGTGAAGACCTCTG  | 146            |
| SERPINE1_F  | TTGCAGGATGGAACTACGGG           | 100            |
| SERPINE1_R  | GTGGCAGGCTGACGACAGATT         | 100            |
| SMAD3_F     | CTCTTCTCTTGGCCGTCGGGA          | 124            |
| SMAD3_R     | GCCCAAACCTTCGCTCAACT           | 124            |
| SNAI2_F     | ACAGCAGAATGGACGACACAT          | 130            |
| SNAI2_R     | CGGTTAGTCCACACAGTGT           | 130            |
| SRC_F       | ATCACCAGACGGGAGTCAGA          | 98             |
| SRC_R       | CAGTAGGCGACCTTTCTGAGT         | 98             |
| TEAD1_F     | TTTGCCACGTTGTGGTTTGC          | 100            |
| TEAD1_R     | ACGCTTACATCCACAAGCCCA         | 100            |
| TEAD2_F     | TACATCAAGCTGAGAACGGG          | 106            |
| TEAD2_R     | CTTCAACCTTGGAAGTGGATTT        | 106            |
| TGFB1_F     | AGGATCTCTCCTCTCGACCT          | 111            |
| TGFB1_R     | GACCGGGGGGTGTCAGAT           | 111            |
| TGFB2_F     | GTTACAACACCTCCTCTGGCTA        | 96             |
| TGFB2_R     | TCTGTAGAAAGTGGGCGCGGA         | 96             |
| TGFB3_F     | AACCACGCTACCTGTGGATT          | 80             |
| TGFB3_R     | TATGGCAACCTCAGGCTGGTG         | 80             |
| TIAM1_F     | CCCCCCTCCAGGAAACCTGAAC        | 119            |
| TIAM1_R     | GGGACATTTCTTGTGACGG          | 119            |
| VAV3_F      | CATTGTCACGGGCCTGTTG           | 150            |
| VAV3_R      | TGATTCTCTGTTGGCAATGCTA        | 150            |
| YWHAB_F     | TCCCAATGTGCTACAAACAGGAA      | 121            |
| YWHAB_R     | CTGGGAGTTCACACAGTGG          | 121            |
| ACTB_F      | CATGTACGTTTCTATCCAGGC         | 249            |
| ACTB_R      | CTCTTTGATGTCACGCAGAT         | 249            |
| Patient # | Biochemical recurrence | CTC number collected and analyzed | Gleason score | PSA levels when CTCs were collected |
|-----------|------------------------|----------------------------------|---------------|-----------------------------------|
| VTC010    | -                      | 29                               | 8             | 125.98                            |
| VTC004    | -                      | 18                               | 7             | 6.31                              |
| VTC046    | -                      | 7                                | 7             | 0.24                              |
| VTC034    | +                      | 19                               | 8             | 1306.65                           |
| VTC048    | +                      | 6                                | Unknown       | 0.16                              |
| VTC035    | +                      | 12                               | 8             | 0.5                               |
| VTC043    | +                      | 23                               | Unknown       | 3.48                              |
| VTC047    | +                      | 8                                | 7             | 0.24                              |
| VTC027    | +                      | 14                               | 7             | 37.17                             |

Table S3 | Summary of patients' pathological status and CTCs collected.
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