Identification of Tumour Initiating Cells by a Small Molecule Fluorescent Probe through Vimentin as the Biomarker

Yong-An Lee[1*], Jong-Jin, Kim[1*], Jungyeol, Lee[2], Srikanta Sahu[1], Hwa-Young Kwon[2], Sung-Jin Park[1], Jia Hui Jane Lee[3,4], Se-Young Jang[5], Jun-Seok Lee[5], Zhenxun Wang[3], Wai Leong Tam[3,6,7], Bing Lim[3†], Nam-Young Kang[1*], Young-Tae Chang[1,2,8*]

[1]Laboratory Bioimaging Probe Development, Singapore Bioimaging Consortium, Singapore 138667, Singapore

[2]Department of Chemistry, Pohang University of Science and Technology, Pohang 37673, Republic of Korea

[3]Stem Cell and Developmental Biology, Genome Institute of Singapore, Singapore 138672, Singapore

[4]School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore

[5]Molecular Recognition Research Center, Korea Institute of Science and Technology, Seoul 02792, Republic of Korea

[6]Cancer Science Institute of Singapore, National University of Singapore, Singapore 117599, Singapore

[7]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

[8]Center for Self-assembly and Complexity, Institute for Basic Science (IBS), Pohang 37673, Republic of Korea

[*] These authors contributed equally to this work.

[†]Current address: Merck Sharp and Dohme Translational Medicine Research Centre, Singapore

Correspondence and requests for materials should be addressed to N.-Y.K. (kang_nam_young@sbic.a-ster.edu.sg) or Y.-T.C. (ytchang@postech.ac.kr).

Tumour initiating cells (TICs) has been alleged for clinical relapse and metastasis in a variety of epithelial cancers including lung cancer[1]. While the efforts on development of probe for TIC have been continued for their detection and targeting[2-4], the universal TIC probe is yet remained to be developed. Here we report the first TIC specific fluorescent chemical probe, TiY, with the identification of the molecular target as vimentin, a marker for epithelial-to-mesenchymal transition (EMT)[5-8]. TiY selectively stains TIC over differentiated tumour cells or normal cells, and facilitate the visualization and enrichment of functionally active TICs from patient derived tumour. At high concentration, TiY also showed anti-TIC activity with low toxicity to other cell types. With the unexplored target, vimentin, TiY casted the possibility of the first universal TIC selectivity in many different cancer cell types.

TIC has been suspected to be responsible for the relapse and metastasis of cancers. While various cancer cell surface markers and their selective antibodies have been developed, the scope of each probes are limited to a certain range of TIC, lacking a universal probe for all the cancer types. We have invented diversity-oriented fluorescent library approach (DOFLA), and demonstrated various cell type selective chemical probes through unbiased phenotypic screening[9-11]. While DOFLA is a promising approach for TIC probe development, the unbiased screening requires a reliable enriched TIC cell culture, which has been challenging to set up due to their dynamic population change during the cell culture[12]. Fortunately, we could establish a high purity TIC model cell line of non-small cell lung cancer (NSCLC) derived from patient tumour sphere 32 (TS32), which was enriched by CD166 surface marker[13]. Cells from TS32 have retained high CD166 expression along with the ability of TS forming in a serum free media containing EGF and bFGF[13]. As the counter part of the

TIC line, we differentiated TS32 cells by withdrawing the growth factors from the media and collected CD166 negative adherent type cells (32A; Extended Data Fig. 1a, b). Thousands of DOFL compounds[14] were screened against these two cell lines for a stronger staining signal for TS32 over 32A cells as the primary screening. Different lung TS line (TS10) and normal lung epithelial cells (LuEpi) were used as the additional positive and negative control during the secondary screening, respectively (Extended Data Fig.1c, left). From the several rounds of imaging based screening and flow cytometry analysis, we narrowed down the hit compounds to less than 10, which displayed strongest preferential selectivity to both TS lines over 32A and LuEpi (Fig. 1a, b). For further validation as the TIC probes, the hit compounds were also tested in the FACS purification of TS 32 cell derived xenograft (TDX 32) tumour. The brightly (+) and dimly (-) stained cell populations were compared for their TS forming ability, as an in vitro surrogate assay for assessing self-renewal and tumourigenic capacity (Extended Data Fig. 1c, right) [5]. From the validation step, we identified the most selective compound both by the selective staining and TS forming ability, and dubbed **T**umour **i**nitiating cell probe **Y**ellow (TiY) (Fig. 1c, Supplementary Information). In TS forming performance test, TiY displayed even better selectivity to tumourigenic cells compared to CD166 (Extended Data Fig. 1d-f). TiY stained the cells in the cytosol, in contrast to the surface marker CD166 antibody staining, but they showed a general co-localization in TS (Fig. 1d). The careful dual staining of TS further identified minor populations of $TiY^{+}CD166^{-}$, $TiY^{-}CD166^{+}$ and $TiY^{-}CD166^{-}$ cells. Intriguingly, TiY^{+} cells consistently showed a greater propensity of sphere forming than TiY^{-} cells regardless of CD166 expression (Fig. 1e-g), demonstrating the better TIC recognition of TiY over CD166. To further confirm tumorigenicity of TiY^{+} cells, we performed in vivo xenograft model experiment with FACS purified cells from

82 TDX tumours (Extended Data Fig. 2a-c). Subcutaneous transplants of the purified
83 cells clearly revealed the enriched tumourigenic potential in the TiY^+ cells over TiY^-
84 cells. After the cell line experiments, the animal test was further extended to direct
85 lung cancer patient derived xenograft (PDX) tumours, as an attempt to confirm TiY's
86 applicability to primary cancer patient samples. The result of transplants clearly
87 showed that tumourigenic cells were consistently enriched in TiY^+ cells from various
88 PDX tumours derived from different patients (Fig. 2a-h). Notably, the limiting
89 dilution transplants of FACS-purified tumour cells showed that the frequency of TICs
90 in the TiY^+ cells was far greater than those in TiY^- and unsorted control cells (Fig. 2i,
91 Extended Data Fig. 3)[15]. Taken together, TiY is capable to distinguish TIC from non-
92 TIC in various tumour cell lines and different patient lung tumours.

93 To identify the binding target of TiY in TIC cells, lysates of TS32 and 32A
94 cells were subjected to SDS-PAGE for a fluorescence scan. TiY was originally
95 designed to carry a chemically reactive chloroacetyl group and potentially can make a
96 covalent binding to the target protein. A strong band (at approximate size of 55 kDa)
97 was detected in TS32 cell lysate, whereas no band was observed in 32A cell lysate
98 (Fig. 3a). The band was digested by trypsin and analyzed by a mass spectrometer[16],
99 giving the identity of the protein as vimentin (Extended Data Fig. 4, Supplementary
100 Table). By RT-qPCR experiment, a higher level of vimentin expression was
101 confirmed in TS32 cells over 32A cells (Fig. 3b) and the vimentin expression and TiY
102 staining also showed a high correlation (Fig. 3c). To validate that vimentin is the
103 responsible target of TiY for the staining phenotype, knockdown of vimentin was
104 carried out on TS32 and diminished TiY signal was observed (TS32-Vim; Fig. 3d-e).
105 In opposite approach, a forced expression of vimentin in 32A cells boosted TiY
106 staining (32A+Vim; Fig. 3g-h), suggesting that vimentin is indeed the functional

target of TiY in the lung TIC cells.

Vimentin has been known as the EMT marker and is related in poorly differentiated tumour[17]. We therefore cultured TS32-Vim and 32A+Vim cells in TS culture condition to confirm the role of vimentin in the tumourigenic capacity. The results showed that sphere forming was directly influenced by regulation of vimentin expression (Fig 3f, i). This was consistent with previous reports of the transition in sphere forming ability by regulation of EMT genes expression[7,18].

Withaferin A (Extended Data Fig. 5a; WFA) is a known vimentin inhibitor and disturbs the dynamics between assembly and disassembly of vimentin filaments by covalent binding to tetrameric vimentin, resulting growth inhibition of vimentin expressing cancer cells[19,20]. Thus, we next explored the possibility of TiY of sharing the binding site in vimentin with WFA. From SDS-PAGE gel and live cell images, TiY-vimentin binding was decreased in a dose dependent manner when WFA was pretreated in cells (Fig. 4a-b), suggesting the identical binding site of TiY and WFA in vimentin. To investigate whether TiY also has affinity to the tetrameric vimentin, purified human vimentin was incubated with TiY in the presence of salt to initiate in vitro tetramerization of vimentin[19,21]. The result interestingly showed that TiY has higher affinity to tetramerized vimentin than monomer counterpart (Extended Data Fig. 5b). While WFA has been used as a cancer drug, a general cytotoxicity to normal cells has been a concern[22]. We checked the anti-tumour activity and general toxicity of TiY in comparison with WFA. Not surprisingly, TiY showed a clear anti-tumour activity at higher concentration, and more interestingly showed a much better selectivity over normal tissue and non-TIC cells, while WFA showed much lower selectivity among them (Fig. 4 c, d). As anticipated, the selective inhibition of TiY on

TS cells also resulted in inhibition of sphere formation in dose dependent manner as well as complete inhibition was shown in high concentrations (Extended Data Fig. 5c, d).

The expression level of vimentin is also reported to be in high correlation with aggressive epithelial tumours and invasive cancer cell lines[23-26]. We evaluated the potential of TiY as a universal probe that is applicable to identify and enrich the TICs in various types of cancer. To this end, TiY staining based cell sorting was expanded to various types of human cancer cell lines (lung, CNS, melanoma, breast, renal, ovarian, colon and prostate cancer) of the NCI 60. Surprisingly, TiY^+ cells showed higher sphere forming ability in all the tested cancer cells, compared to unsorted control and TiY^- cells (Fig. 4e-g, Extended Data Fig. 6a-c and e-g). Consistent with the target ID data, TiY^+ cells in all tested cell lines also showed their higher expression level of vimentin compare to TiY^- cells by dual staining of TiY and the antibody for vimentin protein (Fig. 4h, Extended Data Fig. 6d and h).

Collectively, we have discovered a small molecule TIC probe TiY with its binding target as vimentin. Our work demonstrates that TiY selectively stains TICs not only in lung cancer tumour, but also different types of cancer cell lines indicating the potential universality of TiY as TIC probe in various cancers. At high concentration, TiY also showed a selective anti-TIC activity over non-TIC and normal tissue cells. As the first fluorescent TIC probe, TiY would be a valuable tool for visualization and isolation of TICs and also for the applications in non-relapsing cancer drug development targeting vimentin.

1. Vermeulen, L., de Sousa e Melo, F., Richel, D. J. & Medema, J. P. The developing cancer stem-cell model: clinical challenges and opportunities. *Lancet Oncol.* **13,** e83-89 (2012).
2. Magee, J. A., Piskounova, E. & Morrison, S. J. Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell* **21,** 283–296 (2012).
3. Liu, Y. *et al.* Lack of correlation of stem cell markers in breast cancer stem cells. *Br. J. Cancer* **110,** 2063–2071 (2014).
4. Clark, D. W. & Palle, K. Aldehyde dehydrogenases in cancer stem cells: potential as therapeutic targets. *Ann Transl Med* **4,** 518 (2016).
5. Pattabiraman, D. R. *et al.* Activation of PKA leads to mesenchymal-to-epithelial transition and loss of tumor-initiating ability. *Science* **351,** aad3680 (2016).
6. De Craene, B. & Berx, G. Regulatory networks defining EMT during cancer initiation and progression. *Nat. Rev. Cancer* **13,** 97–110 (2013).
7. Mani, S. A. *et al.* The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133,** 704–715 (2008).
8. Morel, A.-P. *et al.* Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS ONE* **3,** e2888 (2008).
9. Im, C.-N. *et al.* A fluorescent rosamine compound selectively stains pluripotent stem cells. *Angew. Chem. Int. Ed. Engl.* **49,** 7497–7500 (2010).
10. Yun, S.-W. *et al.* Neural stem cell specific fluorescent chemical probe binding to FABP7. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 10214–10217 (2012).
11. Er, J. C. *et al.* NeuO: a fluorescent chemical probe for live neuron labeling. *Angew. Chem. Int. Ed. Engl.* **54,** 2442–2446 (2015).
12. Gilbert, C. A. & Ross, A. H. Cancer stem cells: cell culture, markers, and targets for new therapies. *J. Cell. Biochem.* **108,** 1031–1038 (2009).

178 13. Zhang, W. C. *et al.* Glycine decarboxylase activity drives non-small cell lung
179 cancer tumor-initiating cells and tumorigenesis. *Cell* **148,** 259–272 (2012).
180 14. Lee, J.-S., Lee, J. W., Kang, N., Ha, H.-H. & Chang, Y.-T. Diversity-oriented
181 approach for chemical biology. *Chem Rec* **15,** 495–510 (2015).
182 15. Hu, Y. & Smyth, G. K. ELDA: extreme limiting dilution analysis for comparing
183 depleted and enriched populations in stem cell and other assays. *J. Immunol.*
184 *Methods* **347,** 70–78 (2009).
185 16. Meng, W. *et al.* One-step procedure for peptide extraction from in-gel digestion
186 sample for mass spectrometric analysis. *Anal. Chem.* **80,** 9797–9805 (2008).
187 17. Nieto, M. A., Huang, R. Y.-J., Jackson, R. A. & Thiery, J. P. EMT: 2016. *Cell*
188 **166,** 21–45 (2016).
189 18. Chang, C.-J. *et al.* p53 regulates epithelial-mesenchymal transition and stem cell
190 properties through modulating miRNAs. *Nat. Cell Biol.* **13,** 317–323 (2011).
191 19. Bargagna-Mohan, P. *et al.* The tumor inhibitor and antiangiogenic agent
192 withaferin A targets the intermediate filament protein vimentin. *Chem. Biol.* **14,**
193 623–634 (2007).
194 20. Thaiparambil, J. T. *et al.* Withaferin A inhibits breast cancer invasion and
195 metastasis at sub-cytotoxic doses by inducing vimentin disassembly and serine 56
196 phosphorylation. *Int. J. Cancer* **129,** 2744–2755 (2011).
197 21. Robert, A., Rossow, M. J., Hookway, C., Adam, S. A. & Gelfand, V. I. Vimentin
198 filament precursors exchange subunits in an ATP-dependent manner. *Proc. Natl.*
199 *Acad. Sci. U.S.A.* **112,** E3505-3514 (2015).
200 22. Sharma, A., Sharma, R., Chaudhary, P., Dobhal, M. P. & Sharma, M. C. Selective
201 cytotoxicity of non-small cell lung cancer cells by the Withaferin A-fortified root

extract of Ashwagandha involves differential cell-cycle arrest and apoptosis. *Phytopharmacology* **1,** 54-70 (2011).

23. Liu, L.-K. *et al.* Upregulation of vimentin and aberrant expression of E-cadherin/beta-catenin complex in oral squamous cell carcinomas: correlation with the clinicopathological features and patient outcome. *Mod. Pathol.* **23,** 213–224 (2010).
24. Hu, L. *et al.* Association of Vimentin overexpression and hepatocellular carcinoma metastasis. *Oncogene* **23,** 298–302 (2004).
25. Wei, J. *et al.* Overexpression of vimentin contributes to prostate cancer invasion and metastasis via src regulation. *Anticancer Res.* **28,** 327–334 (2008).
26. Liu, Z., Brattain, M. G. & Appert, H. Differential display of reticulocalbin in the highly invasive cell line, MDA-MB-435, versus the poorly invasive cell line, MCF-7. *Biochem. Biophys. Res. Commun.* **231,** 283–289 (1997).

Supplementary Information is available in the online version of the paper.

Author contributions Y.-T.C. conceived and designed the project. Y.-T.C. and N.-Y.K. designed and supervised the experiments. Y.-A.L. designed, performed experiments, analyzed data and wrote manuscript. J.-J.K. designed, performed experiments and analyzed data. J.L. invented probe and analyzed chemical properties of probe and wrote chemistry part of manuscript. S.S. invented probe. H.-Y.K. performed experiments and contributed to data analysis. S.-J.P. contributed to experiment designs and data analysis. J.H.J.L contributed to work on PDX samples. S.-Y.J. and J.-S.L. contributed to work on the target identification study. J.H.J.L and Z.W contributed to the maintaining cell culture and PDX. W.L.T provided the PDX samples and contributed to design of PDX study. B.L conceived and designed the project. All authors discussed the results and commented on the manuscript.

Author information Correspondence and requests for materials should be addressed to Nam-Young Kang or Young-Tae Chang. Yong-An Lee and Jong-Jin, Kim contributed equally to this work as main authors.

Figure 1 | Discovery of fluorescent probe for Lung TIC. a, Fluorescence images of TS32, TS10, 32A and LuEpi cells with TiY. **b**, Flow cytometric analysis of TS32, TS10, 32A and LuEpi cells stained with TiY. **c**, Chemical structure of TiY. **d,** Expression of CD166 at TiY-stained cells in TS32 and TS10. **e-g,** Quantification of tumour sphere formation of cells collected from TS cells after dual staining of TiY and antibody for CD166. Values are means ± SEM (n = 4).). scale bar = 400 μm (a), 25 μm (d), 500 μm (g).

Figure 2 | Assessment of TiY as a TIC probe in tumour. Comparison of tumorigenicity among the Con, TiY+, and TiY- cell populations in PDX A233 (50,000 cells injection; a-c), A234 (20,000 cells injection; d-f) and A139 (5,000 cells injection; g, h). In case of the A139-tumour, more diluted cell numbers (1000 – 1 cells) were injected for the extreme limiting dilution analysis to confirm the enriching efficiency of TICs in TiY+ cells from the tumour cells (i). Values are means ± SEM (n = 8). Total number of mice and sites in a mouse injected the tumour cells were 4 and 2, respectively). Scale bar = 1 cm.

Figure 3 | Identification of TiY Binding Protein. a, Fluorescence scan of gel separated by 1D SDS-PAGE of protein lysate from TiY-stained 32A and TS32 cells. Mass spectrometer analysis indicated human vimentin as a major protein of the band. **b,** Comparison of vimentin expression level using RT-qPCR between 32A and TS32 cells. **c**, Flow cytometric quadrant dot plot of 32A and TS32 cells after dual staining of TiY and antibody for vimentin. **d,** RT-qPCR data for comparison of vimentin expression level between TS32 treated transfection agent and empty vector (TS32-

Mock) and siRNA-mediated cells for knockdown of vimentin (TS32-Vim). **e,** Fluorescence microscope images after staining TiY in TS32-Mock (left) and TS32-Vim (right) cells. **f,** Comparison of sphere forming ability between TS32-Mock and TS32-Vim cells. **g**, RT-qPCR data for comparison of vimentin expression level between 32A treated with empty vector (32A+Mock) and vimentin overexpression vector (32A+Vim) with transfection agent. **h,** Fluorescence microscope images after staining TiY in 32A+Mock (left) and 32A+Vim (right) cells. **i**, Comparison of sphere forming ability between 32A+Mock and 32A+Vim. Values are means ± SEM (n = 3). Scale bar = 50 μm (e and h), 400 μm (f and i).

Figure 4 | Assessment of universality of TiY. a-b, Binding competition assay of TiY. Fluorescence microscope images for binding competition of TiY with WFA in TS32 (a). Fluorescent scan for TiY and vimentin in SDS-PAGE gel and Western blotting, respectively (b). **c-d,** Comparison of cytotoxicity of TiY (c) and WFA (d) in two TS lines, 32A and normal lung epithelial cells (LuEpi). **e-g,** Quantification of tumour sphere formation of cells collected by TiY dependent cell sorting for unsorted control (Con), brightly stained (TiY+) and dimly stained cells (TiY-), in different types of cancer cells from NCI-60. **h,** Flow cytometric quadrant dot plot of different cancer cells after dual staining of TiY and antibody for vimentin. Values are means ± SEM (n = 3). scale bar = 100 μm (a), 500 μm (g).

Extended Data Figure 1 | TiY has selectivity to TICs in CD166+ TS cells. a, Schematic process of derivation of 32A cells from TS32 cells. **b,**.Comparison of CD166 expression level between TS32 and 32A cells. **c,** Schematic diagram of the

experimental procedure for discovering fluorescent probe for lung TIC. TiY was discovered from screening and validation steps. TICs, TS32 and TS10. non-TICs, 32A (adherent type cells derived from TS32 cells) and human normal lung epithelial cells (LuEpi). TDX, TS cell derived xenograft. **d-f,** Quantification of tumour sphere formation of cells sorted by TiY or CD166 antibody dependent cell sorting, unsorted control (Con), brightly stained (+) and dimly stained cells (-), in TS32 xenograft (TDX 32) tumour cells. Values are means ± SEM (n = 3). Scale bar = 400 μm.

Extended Data Figure 2 | TiY has selectivity to TICs in TS derived xenograft tumour cells. a, Schematic diagram of the experimental procedure for validation of TiY as a TIC probe. **b-c,** Comparison of the tumorigenicity between three populations in tumour cells, which are unsorted control (Con), brightly (TIY+) and dimly (TiY-) stained cells. TiY dependent cell sorting and injection into mice was conducted in two types of TDX tumour, TS32 derived xenograft (TDX 32; b) and TS10 derived xenograft (TDX 10; c). Values are means ± SEM (n = 6). Total number of mice and sites in a mouse injected the tumour cells were 3 and 2, respectively. Scale bar = 1 cm

Extended Data Figure 3 | Assessment of enriching efficiency of TiY for TICs in tumour cells. Limited cell numbers (1000 – 1 cells) were injected to confirm the enriching efficiency of TICs from the A139 tumour cells. Values are means ± SEM (n = 8. Total number of mice and sites in a mouse injected the tumour cells were 4 and 2, respectively). Scale bar = 1 cm.

Extended Data Figure 4 | The result of protein identification of TiY binding target. a, Schematic diagram of the experimental procedure for identification of TiY binding target. **b,** Fluorescence scan of gel separated by 1D SDS-PAGE of protein lysate from TiY stained TS32 cells (left) and the list of proteins detected from gel bands in white dotted box by protein identification procedure (right; MALDI/MS). The list is summary of the analysis results (Supplementary Data 2). The result indicated that vimentin and SERBP1 would be potential target protein due to their high protein score (protein score C.I > 95 %), which is indicating a non-random event. **c,** Sequence coverage map of vimentin and SERBP1. Vimentin was considered a best potential target as it showed highest protein score (100%) and sequence coverage (52.1%; The bold red in the amino acid sequence indicates the matched peptides detected in the result of the protein ID).

Extended Data Figure 5 | Sphere forming inhibition of TiY. a, Structure of withaferin A (WFA) **b,** Kinetic measurement of fluorescence of TiY stained in testrameric and monomeric vimentin. **c-d,** TS culture with TiY. Values are means ± SEM (n = 3). Scale bar = 400 μm.

Extended Data Figure 6 | TiY has selectivity to TICs in different type of cancer cells. a-c, Quantification of tumour sphere forming of cells collected by TiY dependent cell sorting for unsorted control (Con), brightly stained (TiY^{+}) and dimly stained cells (TiY^{-}) in lung cancer cells from NCI60 cell lines. **d**, Flow cytometric quadrant dot plot of lung cancer cells after dual staining of TiY and antibody for vimentin. **e-g**, Quantification of tumour sphere forming of cells collected by TiY dependent cell sorting in different types of cancer cells from NCI60 cell lines. **h**, Flow

cytometric quadrant dot plot of different cancer cells after dual staining of TiY and antibody for vimentin. Values are means ± SEM (n = 3). Scale bar = 500 μm. Spheres larger than 50 μm were counted in the study.

Methods

Cell cultures. Unless otherwise stated, all reagents for cell cultures were purchased from Thermo Fisher Scientific. Tumour spheres (TS) were grown in serum free condition as described previously[13]. DMEM/F12 containing 15 mM HEPES, 2 mM L-glutamine, 0.6% glucose, 1 mg/ml NaHCO3 (SigmaAldrich), 1 % NEAA, 4 mg/ml BSA (SigmaAldrich), 1 % ITS (Corning), 1% antibiotics. 20 ng/mL EGF and 4 ng/mL basic fibroblast growth factor (bFGF) were supplemented to the media and the cells were cultured in the Petri dishes (BD). Tumour spheres were passaged by Accutase digestion into single cells and re-plated into dishes described above. Human normal lung epithelial cells were (ATCC) and maintained in basal medium containing BSA, BPE, hydrocortisone, epinephrine, transferrin, insulin, retinoic acid, triiodothyro- nine, GA-1000, hEGF. Human cancer cell lines were maintained in RPMI/10% FBS. The lung epithelial cells and cancer cell lines were passaged by trypsin-EDTA digestion and fresh medium was replenished every 3 days for all cell cultures.

DOFL screening for TIC probe development. DOFL compounds were diluted with the culture medium to make a final concentration of 1 μM. The compounds were added in cell culture prepared side by side in 384-well plates seeded positive and negative control cells, respectively. After incubation with DOFL compounds for 1 hour, the fluorescence cell images of each well were taken using a high throughput imaging equipment, ImageXpress Microcellular imaging system (Molecular Device),

and the fluorescence intensity was analyzed based on visual confirmation on images. The compounds which stained TS cells with higher intensity than non-TIC negative controls were selected as hit compounds.

Patient samples and patient derived xenograft (PDX) lines maintenance. All lung cancer patient tumour cells were collected and prepared as described previously[13]. In brief, surgical samples of patients were with cold PBS with antibiotics, chopped with a sterile blade, and incubated in 0.001% DNase (Sigma Aldrich), 1 mg/ml collagenase/dispase (Sigma Aldrich) in DMEM/F12 medium (Thermo Fisher Scientific) at 37°C water bath for 3 hr with intermittent periodic agitation. After incubation, the suspensions were washed by DPBS, passed through 70 mm and 40 mm cell strainers, and centrifuged. Cells were resuspended and evaluated for the viability. For generating patient derived xenograft (PDX) tumours generation, the tumour cells were co-injected with Geltrex (Thermo Fisher Scientific) by subcutaneous injections to NOD/SCID Il2rg$^{-/-}$ (NSG) mice. All PDX tumours were maintained by passages into new NSG recipient mice without cell culture.

Xenograft tumours. For generations TS derived xenograft (TDX) tumours, TS cells were harvested from the TS culture and subcutaneously injected to NSG. The tumour cells were prepared as described above in method for the patient tumour cell preparations.

Flow cytometry and fluorescence-activated cell sorting. Cells were stained with TiY or labeled with antibodies against CD166-PE (R&D systems) in DPBS containing 2% FBS and antibiotics (DPBS-S) for more than 40 min at 4°C. The cells were washed and resuspended in the DPBS-S. The fluorescence intensity was measured on a flow cytometry (BD™ LSR II) or FACS was performed on a

FACSAria II (BD) for cell sorting.

In vivo tumour formation assay. FACS-purified cells were co-injected with Matrigel by subcutaneous injections to NSG mice as described above.

Limiting dilution assay. For limiting dilution assay, single cells were harvested from A139 tumour. Cells were sorted from either the 15% highest or the 15% lowest TiY-intensities dependently. The cells were then diluted into 1, 5, 20, 100, 300 and 1,000 and injected subcutaneously as described above. Same number from unsorted cells were also injected as a unsorted control. TIC frequency was evaluated using the Extreme Limiting Dilution Analysis (ELDA) software (http://bioinf.wehi.edu.au/software/elda/index.html).

Flow cytometric quadrant dot plot of TiY and vimentin antibody. After the single cell dissociation, cells were stained with TiY in suspension at 4°C for more than 40 min. Cells were then 4% PFA-fixed for 10 min, permeabilized in 0.2% Triton-X in DPBS for 5 min and blocked in DPBS containing 2% BSA for 1 hour. The cells were then labeled with the antibody against vimentin-660 in DPBS containing 1% BSA and 0.2% Tween-20 at 4°C overnight. The fixation, permeabilization, blocking and antibody labeling were performed in suspension. Two times washing steps were proceeded between every step and the fluorescence intensities were measured on a FACSAria II (BD).

Immunofluorescence for tumour spheres. TS32 and TS10 were stained with TiY for 1 hr, 4% PFA-fixed for 10 min and blocked in DPBS containing 2% BSA for 1 hour. The spheres were then incubated with the antibody against CD166 in DPBS containing 1% BSA and 0.2% Tween-20 at 4°C overnight. Subsequently, the spheres were incubated with the Alexa Fluor conjugated secondary antibody (Thermo Fisher

Scientific) for 3 hr. The fixation, permeabilization, blocking and antibody labeling were performed in suspension, and the spheres then allowed to settle onto dishes pretreated with poly-D-lysine. Two times washing steps were proceeded between every step and the dishes were viewed on a A1R^{+}si confocal microscope (Nikon) for fluorescent from TiY and CD166.

RNA preparation and real time quantitative PCR (RT-qPCR) analysis. Total RNA was isolated using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instruction. The reverse transcription and amplification were carried out using Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystem) on a StepOne™ Real-Time PCR System (Applied Biosystem) according to the manufacturer's instructions. The relative mRNA levels of the genes of interest were normalized to beta-actin

Tumour sphere formation assay. For TS cells**,** FACS-purified 5,000 cells were cultured in a well of non-culture treated 12 well plate. For cancer cell lines, FACS-purified 200-500 cells were culture in a well of ultra-low attachment plate. The cells were cultured in the plates containing TS culture media at 37 ℃, 5% CO_2 and the number of sphere counted under the microscope after 4-7 days. Spheres were counted if the size is larger than 50 μm.

Vimentin Knockdown and forced expression. For SiRNA mediated vimentin gene knockdown, 100,000 – 200,000 of TS32 cells were prepared in a well of 6 well plate with 1 mL of the TS culture media. The cells were transfected with 1 mL of OPTI MEM containing1RNAi duplex-Lipofectamine RNAiMAX (Thermo Fisher Scientific) complexes according to the manufacturer's instructions. For the vimentin forced expression, 1,000 – 2,000 32A cells were seeded in a well of 6-well plate with

1 mL of culture media. Cells were then cultured for a day and carried out transfection with 1 mL of OPTI MEM including plasmid constructs (Origene) and 10 µL of Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. For both transfections, cells were cultured with the transfection complexes for 48 – 72 hours without medium change. The validation of the transfections by RT-qPCR according to method described above. For observing changes of TiY signal in cells after the transfections, the cell plates were stained with TiY after the transfections and the fluorescence was viewed on a Nikon fluorescent microscope (Ti-E) or Axio Observer (Carl Zeiss).

SDS-PAGE for protein target identification. TiY stained cell pellet was lysed in a lysis buffer (Thermo Fisher Scientific) premixed with 10 µL/mL Protease Inhibitor Cocktail (GE healthcare). The cell extract was homogenized by constant agitation for 30 min, and centrifuged at 16,000 × g for 20 min at 4 °C. The supernatant was collected and protein concentration was determined by Bradford protein assay reagent (Bio-Rad). 30-50 µg of each sample was taken, mixed with 4× Laemmli sample buffer (Bio-Rad) and analyzed by SDS-PAGE after boiling at 95°C for 5 min. To find the desired bands, in gel fluorescence was scanned with a Typhoon (GE healthcare).

MALDI-TOF/TOF MS. To identify protein in desired bands, the residual peptides were extracted by in-gel digestion using trypsin and then subjected to MALDI-TOF/TOF MS[16]. In brief, the tryptic peptide was spotted onto the MALDI target plate according to manufacturer's protocol. The spotted sample was analyzed by an ABI 4800 Proteomics Analyzer MALDI TOF/TOF mass spectrometer (Applied Biosystems) and both MS and MS/MS spectra were recorded and submitted via GPS Explorer (Applied Biosystems) to Mascot server (Matrix Science) for database

searching. The database utilized for the search was the Swissprot considering variable modifications of carbamidomethyl at cysteine (C) and Oxidation at methionine (M) and allowing precursor ion tolerances of < 0.15 Da and MS/MS fragment ion error tolerances of < 0.3 Da.

TS forming inhibition test of TiY. For in vitro test of TS forming inhibition, TS cells were culture with TiY at different concentrations without washout. Number of spheres was counted after culture for 7 days.

TiY cytotoxicity test. Cells in a 96-well plate were treated with different concentrations of TiY or WFA. After 48 h, cells were incubated with 20 μl CCK dye (Dojindo) per well for 2 hr. Absorbance was determined at 450 nm using a fluorescence microplate reader (Molecular Devices). The percentage of cell proliferation was calculated by converting the absorbance to percentage of control.

Data availability. All data that generated during current study are available from the corresponding authors upon reasonable request.