SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Construction of Antibody Phage Display Library, Related to Figure 2.

(A) The constant (C) region of an antibody is relatively conserved, whereas the variable (V) region is antigen-specific. The V region consists of alternating framework (FW) and hyper-variable complementarity-determining regions (CDR). We generated Fv (fragment variable), which is composed of the variable light (V_L) and variable heavy (V_H) regions. In single chain variable fragments (scFvs), the two variable regions are artificially joined with a neutral peptide linker and expressed as a single polypeptide chain [adopted from Amersham Biosciences, 2000]. We used antibody phage display technique to generate functional antibody fragments in the form of scFvs as fusion proteins with a minor phage coat protein pIII. Functional antibody elements were displayed on the phage surface by introducing the antibody gene into engineered recombinant phagemid vector [adopted from Sommavilla, 2010].

(B) Individual clones were selected randomly from the enriched scFv pools generated by the subtractive panning steps and were sequenced and aligned by multiple sequence alignment, MultAlin (http://multalin.toulouse.inra.fr/) (Corpet, 1988). A representation of a multiple sequence alignment of the selected scFvs is presented. All scFvs in this alignment are tested either *in vitro* or expressed as intrabodies to assess their interaction with PTP1B-OX in cells. Individual sequence numbers shaded in green are the scFvs that were found to be inhibitory to PTP1B-OX reactivation or to interact with PTP1B-OX when expressed as intrabodies.

Figure S2. Reversible Oxidation and Reactivation of PTP1B (1-394), Related to Figure 3.

PTP1B (1-394) (15 nM) was reversibly oxidized by H_2O_2 (50 µM to 1 mM), which was removed by buffer exchange. Aliquots of H_2O_2-treated protein (5 nM final) were used in the phosphatase assay without or with TCEP to observe the inactivation and reactivation of the enzyme, respectively.

Figure S3. Analysis of the Binding Interaction between PTP1B-OX/PTP1B-CASA and scFv45 by Surface Plasmon Resonance, Related to Figure 4.

(A) PTP1B-CASA (1 µM) and PTP1B-OX (1 µM) were injected separately on scFv45 (500 nM) and the binding sensograms were compared.

(B) PTP1B-CASA (1 µM) with or without 2 mM TCEP was injected on immobilized scFv45 (500 nM) and binding sensograms were compared.

Figure S4. Position of Residue Differences between PTP1B and TCPTP within the PTP Domain, Related to Figure 5.

In the structure of PTP1B (C215S) in complex with a bis-phosphotyrosine peptide (yellow) [PDB code: 1G1H, (Salmeen et al., 2000)], residues that differ between PTP1B and TCPTP are colored magenta. These occur throughout the PTP domain and are particularly abundant in the α1' and α2' helices and β5-β6 loop in closest proximity to the catalytic site.

Figure S5. Expression of, and Screening for, PTP1B-OX specific scFv Intrabodies in 293T Cells, Related to Figure 6.

Individual scFvs were transiently expressed in 293T cells as intrabodies to screen and identify candidate scFvs that can bind to endogenous PTP1B-OX. Transfected cells were treated with H_2O_2 (1 mM). Reduced samples were prepared by treating cells with NAC (20 mM) and preparing the lysates with 2 mM TCEP in the lysis buffer. Oxidized and reduced cell lysates

(1 mg) were used for Ni-NTA precipitation as indicated. Protein complexes were eluted from the beads and analyzed by immunobloting to detect PTP1B and scFvs as described previously. For all the samples, input lysates (50% of the precipitates) were loaded and endogenous PTP1B and overexpressed intrabody were detected. Numbered scFvs shaded green indicate individual intrabodies that bound to endogenous PTP1B-OX.

Figure S6. scFv45 Enhances and Prolongs PTP1B-Mediated Insulin Signaling in 293T Cells, Related to Figure 6 and 7.

(A) Cells (293T) transiently expressing scFv45 or scFv20 were treated with insulin (25 nM) for the indicated time points and total cell lysate (60 µg) was subjected to immunoblot analysis of the proteins as indicated.

(B) Catalase was ectopically expressed alone or co-expressed with scFv20 in 293T cells, which were treated with insulin (25 nM) for indicated time points. Total cell lysates (60 µg) were subjected to immunoblot analysis. Tyrosine phosphorylation of IRS-1 and IR-β were detected with anti-phospho-tyrosine antibody (4G10) and rabbit polyclonal site-specific anti-phosphotyrosine antibody [pY1162/pY1163]-IR-β, respectively. The extent of catalase overexpression was observed by immunoblotting with anti-catalase antibody.

(C) Cells (293T cells) expressing intrabodies scFv45 or scFv20 were treated with insulin (25 nM) for different times as indicated. Lysates (30 µg) were subjected to immunoblot analysis, as indicated. Tyrosine phosphorylation of IR-β subunit was detected by rabbit polyclonal site specific anti-phosphotyrosine antibody recognizing pYpY[1162/1163] or pY[1328] in IR-β.

Figure S7. Effect of scFv45 Expression on Insulin and EGF Signaling in HeLa Cells, Related to Figure 6 and 7.

(A) HeLa cells transiently expressing scFv45 were treated with insulin (25 nM) for the indicated times. Total cell lysate (60 µg) was subjected to immunoblot analysis of the proteins as indicated.

(B) HeLa cells transiently expressing scFv45 were treated with EGF (100 ng/ml) for different times as indicated and cell lysates (30 µg) were subjected to immunoblot analysis. Tyrosine phosphorylation of EGFR was detected by anti-phospho-tyrosine antibody (4G10). Total EGFR was detected by rabbit polyclonal anti-EGFR antibody. Phosphorylation of MAPK (Erk1/2) was detected by rabbit polyclonal phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody and total p44/42 was detected by rabbit anti-MAPK (Erk 1/2) antibody. Intrabody 45 was immunoblotted with HRP conjugated anti-HA antibody [anti-HA (3F10)-HRP].

Table S1. Crystallographic Statistics and Parameters, Related to Figure 1

Data collection statistics	
a (Å)	88.5
b (Å)	88.5
c (Å)	104.0
Space group	$P3_121$
Resolution (Å)	50-2.80 (2.87-2.80)
Number of unique reflections	11735
Completeness (%)	97.4 (97.7)
I/sI	6.8 (1.5)
Multiplicity	3.17
R_{merge}	0.128 (0.455)
X-ray source and detector (l)	Rigaku/Mar345 (1.54 Å)
Refinement Statistics	
Resolution (Å)	64.33-2.70
Number of reflections	
Working set	11412
Test set	591 (4.9%)
Z	1
R_{work}	0.229
R_{free}	0.262
Protein atoms	2287
Solvent atoms	0
RMSD from ideal	
Bond length (Å)	0.007
Bond angle (°)	1.32
Ramachandran plot statistics	
Preferred (%)	91.3
Allowed (%)	7.9

Data in parentheses corresponds to the highest resolution shell.

EXTENDED EXPERIMENTAL PROCEDURES

Crystallization

PTP1B-CASA was expressed, purified and crystallized as for wild type PTP1B (Barford et al., 1994a). X-ray data were collected in-house to a resolution of 2.8 Å using a Rigaku RU800 X-ray generator and Mar345 area detector, and processed using the HKL program (Minor et al., 2006). The structure was refined using reduced wild type PTP1B as a starting model (PDB code: 2HNQ) (Barford et al., 1994) using CNS (Brunger et al., 1998) and analyzed using COOT (Emsley & Cowtan, 2004).

Construction of scFv Phage Display Library

A phage display library expressing single chain variable fragments (scFvs) fused to its surface protein pIII was constructed by PCR amplification of the immune genes extracted from the spleen and bone marrow of chickens immunized with PTP1B-CASA. All the PCRs were performed using Amplitaq DNA Polymerase (Applied Biosystems). The final scFv PCR products were pooled together and ethanol precipitated.

A modified version of the pComb3XSS phagemid vector was used for the construction of the scFv antibody libraries. This vector contains an amber codon, which allows for expression of soluble antibody fragments in nonsuppressor strains of bacteria, without excising the gene III fragment. The pComb3XSS also contains two peptide tags, the six histidine (H6) tag and the hemagglutinin (HA) tag for purification of soluble proteins and for immunodetection. The library size (expressed as the transformation efficiency of the ligated library construct) was determined by plating the transformed cells on LB/carbenicillin plates. The culture was then grown under the selection of carbenicillin to select for transformed bacterial cells. To induce the production of

functional phage particles by the infected *E. coli* culture, preparation of a filamentous helper phage VCSM13 (10^{12} PFU/ml) was added and selected with kanamycin. After overnight incubation at 37°C, the phage particles displaying scFv fused to its pIII coat protein were harvested from the supernatant by PEG precipitation.

Subtractive Panning for Isolating PTP1B-CASA Specific Antibody Fragments

A subtractive panning protocol was designed to isolate scFvs selective for the PTP1B-CASA mutant. Different steps of the subtractive panning protocol are described below:

PTP1B Biotinylation *in vivo* and Purification of Biotinylated PTP1B: The biotinylation tag (GLNDIFEAQKIEWHE) (Schatz, 1993) was fused to the N-terminal of PTP1B in pET19b vector and the recombinant protein was co-expressed in *E. coli* [BL21(DE3)] with biotin ligase (pBirAcm). To make the expression construct for NBT-PTP1B (N-terminally biotinylated PTP1B), the 1-321 catalytic domain of the WT or CASA mutant was PCR amplified using forward primer with the entire biotinylation tag with an additional Nco I restriction site and cloned in pET19b plasmid. The primers used for this amplification were–

5´GGGGAACCATGGGCCTGAACGACATCTTCGAGGCTCAGAAAATCGAATGGCAAA ATGGAGATGGAAAAGGAGTTCG3´ (sense) and

5´AGCAGCCGGATCCCCCGGGCTGCAGGAATTCTCTAGACTAGAG3´ (reverse).

Expression of recombinant proteins in bacteria was induced optimally with 1 mM IPTG in the presence of 50 μM biotin in the growth medium for 4 hours at 37°C. The *in vivo* biotinylated PTP1B (both CASA and wild type) was purified in a two-step purification scheme. In the first step the biotinylated recombinant protein was bound to an immobilized Monomeric Avidin support (Thermo Scientific) and then eluted with 5 mM biotin. In the second step the protein was further purified by anion exchange chromatography.

Panning: The scFv library was mixed in solution with 10-50 fold molar excess of wild type PTP1B over the biotinylated PTP1B-CASA under reducing conditions for 4 hours at 4°C to subtract out the pools of antibodies that recognize the common epitopes on both the oxidized and the reduced form of PTP1B. Biotinylated PTP1B-CASA was mixed with this solution and incubated for another 4 hours at 4°C. From this mixture, scFv displaying phage that bound to this biotinylated PTP1B-CASA were captured by streptavidin coated magnetic beads. Non-specific binders were removed by repeated washing. Bound phage displaying specific scFvs on their surface were eluted under acidic (glycine-HCl, pH 2.2) conditions, neutralized and amplified. Amplified phage were used for subsequent rounds of selection and a total of five rounds of panning were performed accordingly. The input phage were pre-incubated with the streptavidin coated beads before each round of panning to eliminate the bead- and streptavidin-binding phage. Input and output phage were estimated to determine whether selective enrichment of specific scFv-displaying phage occurred during the panning steps.

Sequence Analysis of Individual scFv Clones

Randomly selected scFvs from the enriched scFv pools of the subtractive panning steps were sequenced and aligned by multiple sequence alignment, MultAlin (http://multalin.toulouse.inra.fr) (Corpet, 1988) and sorted in groups of identical sequences.

Expression and Purification of Soluble scFvs

Selected scFv clones were expressed under IPTG induction in TOP10F´ *E. coli* and purified with Ni-NTA matrix exploiting the C-terminal His tag.

Phosphatase Assay

Reduced carboxamidomethylated and maleylated lysozyme (RCML) was phosphorylated with [γ^{32}P] ATP using recombinant GST-FER kinase to a stoichiometry up to 0.8 mol ^{32}P incorporated/mol of protein, as described in (Meng et al., 2005). PTP1B preparations (5 nM or as indicated) were mixed with ^{32}P-RCML (100 nM or as indicated) in phosphatase **a**ssay buffer (50 mM HEPES, pH 7.0, 100 mM NaCl, 0.1% BSA, with or without 5 mM TCEP) and incubated at 30°C for 10 minutes. The reaction was stopped with 10% TCA (final) with 2.5% (w/v) BSA, incubated at -80°C for 30 minutes, and thawed at room temperature. The supernatant was collected by centrifugation and the release of ^{32}P-labeled inorganic phosphate was determined by scintillation counting.

Reversible Oxidation of PTP1B by H_2O_2

Purified PTP1B (1-321) or (1-394) (15 nM) was mixed with increasing concentrations of H_2O_2 (0.05 mM to 100 mM) in phosphatase assay buffer (50 mM HEPES, pH 7.0, 100 mM NaCl, 0.1% BSA) at room temperature for 10 minutes. H_2O_2 was removed with a Zeba Desalting Column (Thermo Scientific) equilibrated in the assay buffer without any reducing agent. Phosphatase activity was measured for each protein sample using ^{32}P-labeled, reduced carboxamidomethylated and maleylated lysozyme (^{32}P-RCML) as the substrate, with or without 5 mM TCEP. Activity of each sample was compared to that of the untreated PTP1B in the presence of 5 mM TCEP.

Screening for Conformation Sensor scFvs

We designed an in-solution phosphatase assay for screening the conformation sensor scFvs using ^{32}P-labeled, phospho-tyrosyl RCML as the substrate. Recombinant PTP1B was reversibly oxidized and inactivated with H_2O_2. The phosphatase activity was restored upon the removal of H_2O_2 by a quick buffer exchange and addition of reducing agent (TCEP). Purified bacterially

expressed scFvs (100X molar excess over PTP1B) were incubated with PTP1B-OX and the effect of individual scFv on stabilizing the reversibly oxidized conformation was assessed by the phosphatase assay under reducing conditions. Purified individual scFvs (750 nM) were incubated with PTP1B (7.5 nM) under reducing conditions (5 mM TCEP) in 50 mM HEPES, pH 7.0, 100 mM NaCl, 0.1% BSA for 30 minutes at RT to assess whether the scFvs bound to the reduced form of PTP1B and had any direct inhibitory effect on phosphatase activity. To determine the effect of scFv binding on the reactivation of PTP1B-OX, reversibly oxidized PTP1B (7.5 nM) was incubated with individual scFvs (750 nM) for 30 minutes at room temperature in the same binding condition without reducing agent. PTP1B activity in each sample was determined by RCML phosphatase assay using 100 nM ^{32}P-RCML as the substrate with or without 5 mM TCEP.

Ni-NTA Pull-down Experiments

In order to demonstrate direct interaction between PTP1B-OX and candidate scFvs, we performed an *in vitro* binding assay using purified recombinant 37 kDa PTP1B (residues 1-321, WT or CASA) and purified scFvs under both oxidizing and reducing conditions. Purified PTP1B (50 nM) was reversibly oxidized with 250 µM H_2O_2 followed by a buffer exchange to remove H_2O_2. Purified scFv was incubated in molar excess (100 X) with PTP1B-OX or PTP1B-CASA or with PTP1B under reducing condition (with 2 mM TCEP) in binding buffer (20 mM HEPES, pH 7.4, 300 mM NaCl, 0.05% BSA, 0.05% Tween-20 and 10 mM imidazole) for 2 hours at 4ºC. Ni-NTA agarose (Qiagen, 50 µl as a 50% slurry equilibrated in the binding buffer) was added and incubated for one hour at 4ºC. Protein complexes bound to Ni-NTA agarose beads were precipitated and washed (three times, 5 minutes each, at 4ºC) with binding buffer containing 20 mM imidazole. The protein complexes were eluted from the Ni-NTA agarose beads with 500 mM imidazole (in binding buffer) for 15 minutes at 4ºC with gentle shaking. The complexes were

separated by SDS-PAGE and PTP1B was detected with anti-PTP1B (FG6) antibody and scFv was detected with anti-HA antibody [anti-HA (3F10)-HRP] by immunoblotting.

Surface Plasmon Resonance (SPR)

We used the Surface Plasmon Resonance (SPR) technique for affinity measurements and determination of the kinetic constants for the binding interaction between scFv45 and PTP1B (oxidized, reduced or CASA mutant). Binding interactions in real time were analyzed by BIAcore 2000 system (GE Health Science) using an NTA sensor chip (#BR-1004-07, GE Health Science). All the binding interactions were performed in 0.01 M HEPES, 0.3 M NaCl, 50 µM EDTA, 10 mM Imidazole, 0.05% Tween-20, pH 7.4 as the running buffer. We used the two flow path mode (FC2, FC1) and selected FC1 (Flow Cell 1) as the reference flow cell for determining the background binding and FC2 (Flow Cell 2) as the binding flow cell on which the scFv-PTP1B interaction took place. Both flow cells of the chip were coated with 0.5 mM $NiCl_2$ and washed. A His-HA peptide (HHHHHHGAYPYDVPDYAS, 500 nM) was injected on the reference flow cell (FC1) and purified scFv45 (500 nM) was injected on the binding flow cell (FC2). Following a quick wash, 0.1% (w/v) purified BSA was injected on both flow cells as a blocking buffer to minimize background binding of the proteins to the chip and in the integrated fluid system (IFC). Immediately before the experiment, purified PTP1B was reversibly oxidized with H_2O_2, as described previously, and H_2O_2 was removed by buffer exchange. Increasing concentrations (0.05 µM to 10 µM) of PTP1B-OX were injected on both flow cells and the interaction with scFv45 was observed for 10 minutes. All the reagents and protein samples were prepared in the running buffer, unless stated otherwise, and 20 µl of solute, at 20 µl/minute, was used for each injection. After each round of PTP1B-OX binding, the chip was regenerated by first stripping with 350 mM EDTA, pH 8.3 in running buffer and then by coating with $NiCl_2$. For different PTP1B-OX concentrations or different redox conditions, each sample was injected every time

after stripping and regeneration of the chip followed by freshly immobilizing the scFv on the chip. The binding sensograms were analyzed with the BIAevaluation 3.2 software.

Reversible Oxidation of TCPTP *in vitro* and HA Pull-down Experiment

Bacterially expressed recombinant TCPTP (1-317-6His) was reversibly oxidized by H_2O_2 using identical buffer composition and experimental conditions as for PTP1B. Increasing amounts of H_2O_2 (75 – 200 µM) were added to TCPTP (15 nM) and incubated for 10 minutes at room temperature. After removing H_2O_2 by buffer exchange, oxidized samples were reactivated by TCEP (5 mM). Phosphatase activity was determined using 100 nM ^{32}P-RCML and 5 nM of enzyme from each sample. The activity of each sample was compared to that of the non-treated reduced enzyme, which was set as 100%. To see the effect of scFvs that showed inhibition of PTP1B reactivation, purified scFvs (750 nM) were incubated with oxidized TCPTP and phosphatase assay was determined in presence of TCEP (5 mM). To determine whether scFv45 binds to TCPTP either under reducing condition or when it is oxidized an HA-agarose pull down experiment was performed. TCPTP (50 nM) was oxidized with H_2O_2 (250 µM), which was removed immediately with buffer exchange. An aliquot of oxidized TCPTP (5 nM) was incubated with 500 nM of purified scFv45 in binding buffer (20 mM HEPES, pH 7.4, 300 mM NaCl, 0.05% BSA, 0.05% Tween-20) for 2 hours at 4ºC. Anti-HA monoclonal antibody (Clone 3F10, #11815016001, Roche) conjugated to agarose beads (50 µl of 50% slurry in binding buffer) was added and incubated for an additional hour at 4ºC. Agarose beads were washed 3X with binding buffer and samples were prepared in SDS-sample buffer. Equivalent amounts (4ng of TCPTP) of input, supernatant and precipitate were analyzed by immunoblot. A parallel pull down was performed with oxidized PTP1B and scFv45 using anti-HA-agarose beads as a control. TCPTP was detected with a catalytic domain specific anti-TCPTP rabbit polyclonal antibody (1910H) (Lorenzen et al., 1995).

Expression of Intrabody in Mammalian Cells

To express scFvs as intrabodies, at first scFv45 was cloned into a pCDNA3.2/V5-GW/D-TOPO expression vector for transient expression of the PTP1B-OX intrabody in mammalian cells. The scFv45 sequence from the pCom3XSS phagemid construct was PCR amplified and cloned directionally in the pCDNA3.2/V5-GW/D-TOPO vector. This intrabody was transiently transfected in 293T cells using Fugene 6 as the transfection reagent. Transfected cells were lysed in modified RIPA buffer [25 mM HEPES, pH 7.5, 150 mM NaCl, 0.25% Deoxychloate, 10% Glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1% TritonX-100, 0.5 mM PMSF, 10 mM Benzamidine, Complete protease inhibitor cocktail (Roche)]. Soluble fractions from the samples were blotted with HRP conjugated anti-HA antibody to detect the expressed scFv.

Interaction between PTP1B-OX and scFvs in Mammalian Cells

To detect PTP1B-OX and scFv45 interaction in mammalian cells, scFv45 was overexpressed in 293T cells and 48 hours post transfection the cells were serum-starved for 16 hours in growth medium (DMEM, without serum). Cells were then treated with 25 nM insulin (human insulin, Calbiochem) for 10 minutes, with 1 mM H_2O_2 (in growth medium without serum) for 5 minutes or with 20 mM NAC (in growth medium without serum) for 1 hour, at 37ºC. Cells treated with insulin and H_2O_2 were washed twice with cold (4ºC) PBS and lysed in degassed lysis buffer [25 mM HEPES, pH 7.4, 150 mM NaCl, 0.25% deoxychloate, 1% TritonX-100, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and Complete protease inhibitor cocktail (Roche)]. Cells treated with NAC were lysed with buffer containing 2 mM TCEP to ensure a post-lysis reducing environment. The interaction between PTP1B and scFv45 was tested by precipitating the protein complexes from 1 mg of total cell lysate with Ni-NTA agarose or by immunoprecipitating scFvs with anti-His mouse monoclonal antibody. For the pull down experiment, Ni-NTA agarose beads (50 µl of 50% slurry in lysis buffer) were incubated with the lysates (both oxidized and reduced) at 4ºC for 2 hours. Protein complexes bound to the Ni-NTA agarose beads were

precipitated and washed three times; first with lysis buffer containing 20 mM imidazole followed by two more washes with wash buffer (PBS, 20 mM imidazole pH 7.4, 0.05% BSA, 0.05% Tween-20 and protease inhibitors). The protein complexes were eluted with wash buffer containing 500 mM imidazole with gentle shaking at 4ºC for 15 minutes.

For the immunoprecipitation experiments, 1 mg of total cell lysate was incubated with anti-His mouse monoclonal antibody at 4ºC for 2 hours. The interacting protein complexes were immunoprecipitated after incubating the lysate-antibody mixture with protein A/G Sepharose at 4ºC for 1 hour. After immunoprecipitation, Sepharose beads were washed three times at 4ºC; first with lysis buffer followed by two more washes with wash buffer (PBS, pH 7.4, 0.05% BSA, 0.05% Tween-20 and protease inhibitors). Complexes were separated by SDS-PAGE. PTP1B was detected with anti-PTP1B antibody (FG6) and the intrabodies were detected with anti-HA (3F10)-HRP antibody [anti-HA (3F10)-HRP, Roche] by immunoblotting.

Colocalization of Intrabody 45 and PTP1B-OX

COS1 cells were grown on cover slips and transfected with intrabody constructs of scFv45, or scFv20 as a negative control, using Fugene6 as the transfection reagent. The cells were serum-starved for 16 hours, 24 hours post transfection, at 37ºC and then stimulated with 25 nM insulin or treated with 1 mM H_2O_2 or left untreated. The cells on the cover slips were processed for immune-staining at room temperature. In brief, cells were washed twice with PBS and fixed with 5% formalin for 15 minutes. After washing three times with PBS, cells were permeabilized with 0.5% Triton-X100 for 5 minutes. Permeabilized cells were rinsed with wash buffer [PBS, pH 7.4 with 0.1% BSA, 0.2% TritonX-100, 0.05% Tween-20 and 0.05% sodium azide] and blocked with 5% normal goat serum in wash buffer for 1 hour. To detect endogenous PTP1B and intrabody the coverslips were incubated with a cocktail of anti-PTP1B antibody [rabbit polyclonal anti-PTP1B (H-135), # sc-14021, Santa Cruz Biotechnology] and anti-HA mouse monoclonal

antibody [clone HA.11 (MMS-101R, Covance)] for 1 hour. The cells were washed with the wash buffer to remove excess antibodies. A cocktail of secondary antibodies- Alexa 594 conjugated goat anti-mouse IgG (A-11005, Invitrogen) and Alexa 488 conjugated goat anti-rabbit IgG (Invitrogen, A-11034) was added to the coverslip in the blocking buffer (5% normal goat serum) and incubated in the dark for 1 hour. The coverslips were washed three times with wash buffer and incubated with DAPI (0.3 µg/ml in PBS, #D9542, Sigma) for 10 minutes and washed again with wash buffer. The cover slip was mounted on a glass slide with Vectashield Mounting Medium (H-1000, Vector Laboratories) and observed using a confocal microscope (LSM 710, Zeiss) with a 63X immersion oil objective lens. To quantify colocalization of PTP1B and scFv45, individual images were analyzed by Zeiss (LSM 710) Colocalization Viewer Software. The degree of colocalization of PTP1B and scFv45 in each cell was expressed as colocalization coefficients that measure relative number of colocalizing pixels for the respective fluorophores for PTP1B and scFv45, as compared to the total number of pixels. The numeric range for this colocalization method was set as 0 – 1, where “0” indicates no colocalization and “1” indicates colocalization of all pixels in a cell.

Cell Culture, Sample Processing and Antibodies used for Investigating the Role of scFvs on Insulin Signaling in 293T cells.

Intrabody constructs for scFv45 or scFv20 were transfected in 293T cells using Fugene6 and serum starved 48 hours post transfection. Insulin stimulated cells, with or without the intrabody, were harvested in RIPA buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 0.25% Deoxychloate, 10% Glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1% TritonX-100, 0.5 mM PMSF, 10 mM Benzamidine, protease inhibitor cocktail, 1mM sodium vanadate). Total proteins in the cell lysates were separated by SDS-PAGE and global tyrosyl phosphorylation was detected by anti-phosphotyrosine antibody 4G10 (#05-321, Millipore). To detect specific tyrosyl phosphorylation of IR-β subunit we used rabbit polyclonal anti-insulin receptor [$pYpY^{1162/1163}$ or pY^{1328}] phospho-

specific antibody (#44804G and #44-807G, respectively from Invitrogen). Intrabodies were immunoblotted with anti-HA-Peroxidase, High Affinity (3F10, #12013819001, Roche). For loading controls for corresponding phosphotyrosine proteins, the membrane was stripped by Restore Stripping Buffer (#21059, Thermo Scientific) and re-probed with antibodies against the total protein. We used anti-IR-β rabbit polyclonal antibody (C-19, #sc-711, Santa Cruz) to detect total IR-β subunit and anti-IRS1 rabbit polyclonal antibody (#06-248, Millipore or sc-559, Santa Cruz) to detect total IRS-1. To test the effect of suppressing H_2O_2 levels on intrabody function, catalase was ectopically co-expressed using pS3-Catalase (a gift from Dr. Toren Finkel's laboratory) along with scFv45. Catalase expression was detected in the cell samples with anti-catalase rabbit polyclonal antibody (#219010, Calbiochem). Phosphorylation of the AKT activation loop at residue Threonine 308 (T308) was observed with phospho-specific antibody [phospho-Akt (Thr308), #9275, Cell Signaling]. Total endogenous Akt levels were detected by stripping the membrane and re-probing with Anti-Akt rabbit polyclonal antibody (#9272, Cell Signaling). We measured endogenous PTP1B levels using mouse monoclonal anti-PTP1B antibody (FG6) in all the experiments.

Cell Culture, Sample Processing and Antibodies used for Investigating the Role of scFv45 on EGF Signaling in HeLa cells

HeLa cells were transfected with intrabody constructs encoding scFv45 using Fugene6 (Roche) and 48 hours post-transfection, the cells were serum-starved for 16 hours. Cells were then stimulated with EGF (100 ng/ml) for different times, washed with cold (4ºC) PBS, and harvested in RIPA buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 0.25% Deoxychloate, 10% Glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1% TritonX-100, 0.5 mM PMSF, 10 mM Benzamidine, protease inhibitor cocktail, 1mM sodium vanadate). Total proteins in the cell lysates were separated by SDS-PAGE and tyrosyl phosphorylation was detected by anti-phosphotyrosine antibody 4G10 (#05-321, Millipore). Phosphorylation of MAPK (Erk1/2) was detected by

phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (#4377, Cell Signaling). For loading controls of corresponding phosphotyrosine proteins we used anti-EGFR polyclonal antibody (#2234, Cell Signaling) to detect total EGFR and anti-MAPK (Erk 1/2) antibody (#4695, Cell Signaling) to detect total p44/42 MAPK (Erk1/2). Intrabodies were immunoblotted with anti-HA-Peroxidase, High Affinity (3F10, #12013819001, Roche).

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