Profiling Protein Posttranslational modifications

Antibodies Immobilized as Arrays to Profile Protein Post-translational Modifications in Mammalian Cells

Stanimir S. Ivanov¹*, Alicia S. Chung²*, Paul Z. Yuan³, Angie, Y. Guan³, Katherine V. Sachs¹, Jonathan S. Reichner³, and Y. Eugene Chin¹,²,³**

¹ Department of Pathology & Laboratory Medicine, Brown University Medical School, Providence, RI, 02912; ²Department of Molecular Biology, Cell Biology and Biochemistry, Brown University Medical School, Providence, RI 02912; ³ Department of Surgery, Division of Surgical Research, Rhode Island Hospital and Brown University Medical School, Providence, RI 02912

*Equally contributed authors.

**Corresponding author (y_eugene_chin@brown.edu). Tel. (401) 444-0172; Fax. (401) 444-3278.

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Abbreviations: ankyrin repeats and SOCS box, ASB; near infrared, IR; Polyvinylidene Fluoride, PVDF; suppressor of death domain, SODD; signal transducer and activator of transcription, STAT; Trichostatin A, TSA; ubiquitin, Ub.
Summary: Previously we demonstrated that antibodies printed on a solid support were able to detect protein-protein interaction in mammalian cells. Here we further developed the antibody array system for detecting proteins with various post-translational modifications in mammalian cells. In this novel approach, immuno-precipitated proteins were labeled with fluorescent dye followed by incubation over antibody arrays. Targeted proteins, captured by the antibodies immobilized on polyvinylidene fluoride (PVDF) membrane or glass slide, were detected by means of near infrared fluorescent scanner or fluorescent microscopy. To demonstrate the application of the antibody arrays in protein post-translational modifications, we profiled protein tyrosine phosphorylation, ubiquitination, and acetylation in mammalian cells under different conditions. Our results indicate that antibody array technology can provide a powerful means of profiling a large number of proteins with different post-translational modifications in cells.
Protein function and half-life are often under the tight regulation of post-
translational modifications such as phosphorylation, ubiquitination, and acetylation. Tyrosine phosphorylation is a key mechanism for the reversible regulation of protein activity during signal transduction. The polyubiquitination-proteosome pathway plays a substantial role in the degradation of regulatory proteins in a variety of cellular processes. As for acetylation, accumulating evidence indicates that transcriptional regulation is strongly influenced by the acetylation of histones as well as transcription factors. For many proteins, multiple post-translational modifications may occur simultaneously or sequentially. In the case of p53 tumor suppressor, ubiquitination, phosphorylation, and acetylation, can all affect its activity. Altered protein tyrosine phosphorylation, protein ubiquitination, and protein acetylation in mammalian cells are closely related to developmental diseases and cancer. Recently, mass spectrometry has been employed for identification of proteins or peptides or more specifically, the residues bearing phosphorylation/acetylation modifications. However, a convenient and reliable biochemical method for identifying proteins with the aforementioned post-translational modifications as they occur within a cell lysate is still lacking.

We recently immobilized 50-100 different antibodies on a PVDF membrane for identification of protein-protein interactions in mammalian cells, because antibodies when immobilized on a solid support maintain their binding specificity to target proteins in cells. Meanwhile, purified recombinant proteins or synthesized peptides attached covalently to a glass slide were reported by other labs to be useful in detecting the interaction specifically with other proteins or with small molecules. However, such designed recombinant protein or peptide arrays are mainly used for detecting in vitro protein-protein interactions. Whereas, antibody array technology can be employed to detect both in vitro and in vivo protein-protein interaction.

The current study was undertaken to determine whether antibody array technology could be used to profile protein expression and characterize changes in post-translational modifications that accompany treatment of cells with growth factors, cytokines or drugs.
Results demonstrate an expanded application of the use of antibody arrays for profiling proteins with alterations in tyrosine phosphorylation, ubiquitination, and acetylation in mammalian cells in response to various treatments.

**Experimental Procedures:**

**Cell cultures and antibodies.** A431, HeLa, and 293T cell lines were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) with 10% fetal bovine serum (Invitrogen Carlsbad, CA) with penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37 °C in 5% CO₂ and 95% air. Both 3T3 fibroblasts from STAT1⁺/⁺ and STAT1⁻/⁻ mice⁹ (a kind gift from David Levy) were also maintained in under the same conditions. Whole cell extracts were prepared with RIPA buffer according to the commercial protocol of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). RIPA buffer contained 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTT, with 1 mM PMSF, 1 µg/ml each of Aprotinin, leupeptin and pepstatin, 1 mM Na₃VO₄, and 1 mM NaF. The Bio-Rad (Hercules, CA) protein assay was used to measure protein concentrations and 0.5-1 mg of whole cell extracts was used to setup immunoprecipitation utilizing our published protocol⁴⁵. All antibodies tested here were purchased from Santa Cruz Biotechnology, Inc. except anti-acetyl-lysine polyclonal antibody, which was obtained from Upstate USA, Inc (Charlottesville, VA).

**Fluorescent staining of immunoprecipitates.** The above-prepared whole cell extracts (0.5 - 2 mg) were incubated with antibodies coupled to protein G/A-agarose beads (Santa Cruz Biotechnology, Inc.) for immunoprecipitation. After incubation overnight at 4°C, anti-pY20, anti-Ub, or anti-acetyl-lysine immuno-precipitates were extensively washed with RIPA buffer for 5 - 8 times and re-suspended in 100 µl RIPA prior to fluorescent staining according to the manufacturer’s directions (Molecular Probes, Eugene, OR). In brief, 7.5µl of 2 mg ml⁻¹ Alexa₆₈₀ (excitation/emission maxima of 679/702 nm) obtained from Molecular Probes, or 2 mg ml⁻¹ of Cy2 (489/506 nm) or Cy5 (650/667 nm) obtained from
Amersham Biosciences (Piscataway, NJ) was added to incubate with the immuno-precipitates for 2 - 3 hrs at 4°C. After washing with RIPA for three times, fluorescent-labeled immuno-precipitates were resuspended in 1 ml RIPA buffer and boiled for 5 min prior to incubating with antibody arrays.

**Antibody array preparation and analysis.** On a PVDF membrane obtained from Bio-Rad, commercial antibodies were spotted manually in a volume of 0.4 µl or less (20 – 40 ng) to yield spots less than 500 µm in diameter. When poly-L-lysine coated glass slides (CEL & Associates, Los Angeles, CA) were used as the support, antibodies (2 - 4 ng) were spotted on the top of the slide using the protein-spotter designed by Schleicher & Schuell (Keene, NH), yielding spots less than 200 µm in diameter. It is not necessary to keep antibody arrays hydrated throughout this and subsequent steps. All antibody arrays were stored in sealed plastic bags at 4°C. Antibody arrays were immersed in a PBS buffer containing 3% bovine serum albumin (BSA) for 2 hrs prior to use. Antibody array assays were performed with two distinct methods: (1) Capturing fluorescent proteins: The above fluorescent-labeled protein samples (0.05 - 0.1 mg) were incubated with the above prepared antibody arrays for 3 hrs at room temperature followed by three TBST washes. The fluorescent signals trapped on PVDF membrane arrays were analyzed with the near infrared fluorescent scanner LI-COR Odyssey Fluorescent Scanner manufactured by LI-COR Biosciences (Lincoln, NB). The glass slide arrays were visualized with Nikon fluorescent microscope equipped with appropriate excitation/emission filter sets for each dye. Intensities of the signals were analyzed by spot densitometry on the Odyssey software version 1.1.15. (2) Sandwich assay: Unlabeled protein samples (0.5 - 2 mg) were incubated with the antibody array for 3 hrs at room temperature. After extensively washes with TBST, the antibody arrays were incubated with HRP-conjugated antibody specific for a given post-translational modification (1:1,000 dilution in TBST) for an additional 2 hrs followed by enhanced chemiluminescence (Amersham Biosciences) detection.
**Results:**

**Capturing proteins by antibodies immobilized onto different solid supports.**

Initial experiments were designed to provide proof-of-principle regarding the capability of antibodies immobilized onto PVDF membranes or glass slides to capture and retain their cognate protein antigens from a total cell extracts. After incubation with the extracts of STAT1^{+/+} or STAT1^{-/-} cells labeled with Alexa<sub>680</sub>, the PVDF-antibody arrays were washed and scanned with LI-COR scanner (Fig. 1a). Caspase1 and IRF-1 proteins were detected in STAT1^{+/+} but not in STAT1^{-/-} mouse fibroblast cells (Fig. 1a), in accordance with the previous findings that STAT1 plays an essential role in the expression of these two genes<sup>9-11</sup>. As an additional control, STAT1 was detected in STAT1^{+/+} cells but not in STAT1^{-/-} cells (Fig. 1a).

To investigate whether the micro-antibody array and fluorescent microscopy systems could be useful for detecting proteins, we spotted antibodies on the poly-L-lysine-coated glass slides<sup>12</sup>. Cy2- and Cy5-labeled recombinant His<sub>6</sub>-STAT1 proteins (Fig. 1b) were mixed at different ratios and incubated with a glass slide immobilized with anti-His<sub>6</sub> antibody, anti-STAT1 antibody, or control IgG. The linear color change from green to red visualized with a fluorescent microscope provides a semi-quantitative measure of the proteins that were captured by the anti-His<sub>6</sub> or anti-STAT1 antibody (Fig. 1c). When Cy-2 labeled extracts of HeLa cells were used, fluorescent signals were detected by most of the antibodies that were randomly chosen for printing on the glass slide, although nonspecific interactions between the antibodies immobilized on the slide and the proteins in the sample are not excluded (Fig.1d). Therefore, antibodies attached to either a PVDF membrane or a glass slide retain their abilities to capture cognate ligands from a mixture of cellular proteins and as will be shown below, can then be used for profiling protein post-translational modifications in mammalian cells under different conditions.
Profiling multiple proteins phosphorylated by EGFR in A431 cells.

The antibody array was applied to profile tyrosine phosphorylation in epidermal carcinoma A431 cells. As shown in Fig. 2a and widely reported by others, the activation of EGFR with EGF results in tyrosine phosphorylation of multiple proteins as revealed by western blot analysis using anti-pY20 (Fig. 2a). To identify phosphorylated proteins, pY20 was used to immunoprecipitate tyrosine phosphorylated proteins from extracts of untreated and EGF-treated A431 cells and, after labeling with Alexa680, proteins were incubated with a PVDF antibody array containing 25 antibodies against signaling proteins spotted in duplicate. EGF-dependent tyrosine phosphorylation was evident on AKT and JAK2 (Figs. 2b and 2c). Elevated EGFR phosphorylation in response to EGF treatment was also detected. In A431 cells, EGF has been widely reported to activate STAT proteins and inhibit cell growth. EGF-dependent STAT1, STAT3, and STAT5 phosphorylation (increased 10-fold, 9.5-fold, and 20-fold respectively in Fig. 2c) were clearly visualized in the arrays. Previously, we reported using HRP-conjugated antibody/ECL as a detection method for the antibody array analysis (i.e., sandwich method). These detection systems were directly compared using A431 cell extracts exposed to an antibody array followed by HRP-pY20 immuno-probing. Though less sensitive as compared with the above pY20 immunoprecipitation/fluorescent-labeling assay, pY20-HRP/ECL analysis clearly displayed differential protein phosphorylation patterns between the cells treated with and without EGF (Fig. 2d). Both methods were able to detect tyrosine phosphorylation of EGFR, STAT1, STAT3 and STAT5 in response to EGF treatment in A431 cells (Figs. 2b and 2d).

To demonstrate the specificity of the system for detection of tyrosine phosphorylated proteins, A431 cells were treated with EGF in the presence or absence of the EGFR inhibitor Tyrphostin-AG528. When some antibodies in the array were examined individually, EGF treatment showed an increase in the cellular content of tyrosine phosphorylated EGFR, JAK2 and SOCS3 (Fig. 2e). Tyrphostin-AG528 cotreatment effectively inhibited JAK2 or SOCS3 phosphorylation by EGF, although its effect on EGFR auto-phosphorylation was less effective (Fig. 2e). To confirm the findings resulting from
the antibody arrays in Figs. 2b and 2e, EGFR, JAK2, and SOCS3 were each immuno-
precipitated from extracts of untreated, EGF-treated, and EGF plus Tyrphostin-AG528
treated A431 cells and, their tyrosine phosphorylation levels were confirmed by pY20
immunoblotting (Fig. 2f). Detected with both methods (Figs. 2b and 2d), EGF-dependent
tyrosine phosphorylations of STAT1, STAT3, and STAT5 were confirmed by using
antibodies that recognize specific phosphorylated STAT proteins (Fig. 1g). Thus, the
antibody arrays are capable of identifying multiple pTyr-proteins within a signaling network
such as that downstream of EGFR ligation.

Profiling protein poly-ubiquitination in HeLa cells.

We next applied an antibody array to poly-ubiquitination analysis. TNF-α is a
proinflammatory mediator, which exerts its biological functions by binding two TNF
receptors (TNF-RI and TNF-RII) and inducing protein ubiquitination (Fig. 3a). Whole
extracts prepared from HeLa cells treated, or not, with TNF-α were subjected to anti-Ub
immuno-precipitation. Anti-Ub precipitates labeled with Alexa680 were incubated with the
antibody arrays. Overall more signals were detected in the sample that received TNF-α
treatment (Figs. 3b and 3c), suggesting these proteins were ubiquitinated in response to
TNF-α treatment in HeLa cells. Proteins ubiquitinated by TNF-α treatment and trapped by
the antibody array include TNF-RI, TNF-RII, TRAF2, TRAF6 and SODD, consistent with
the model that ubiquitination modification is an important event during TNF receptor signal
transduction.\textsuperscript{13-15}

Poly-ubiquitinated proteins are short-lived. The proteosome inhibitor MG-132 is
well-known to result in the accumulation of ubiquitin-conjugated proteins. In HeLa cells,
MG-132 caused accumulation of poly-ubiquitinated proteins both in the absence and
presence of TNF-α (Fig. 3d). Consistently, anti-Ub precipitates obtained from HeLa cells
treated with MG-132 alone or MG-132 plus TNF-α contain more SODD and TRAF6
proteins as compared to untreated controls as revealed by the fluorescent signals detected by
their respective immobilized antibodies (Fig. 3e). SODD was identified as a suppressor of
TNF-RI death domain, regulating NF-κB activation. Therefore, SODD dissociation from TNF-RI or SODD deficiency in mouse accelerates NF-κB activation. In HeLa cells, immunoprecipitated SODD proteins were subjected to western blotting analysis with anti-Ub antibody or SODD antibody. SODD ubiquitination was evident at 1 hr of TNF-α administration (Fig. 3f). Moreover, TNF-α-mediated SODD degradation was markedly blocked by treatment with MG-132 (Fig. 3f). Therefore, TNF-α treatment induced SODD ubiquitination and degradation may be responsible for NF-κB activation by TNF-RI.

To apply antibody array for functional study of a novel gene, we screened protein ubiquitination in cells that over-express Ankyrin Repeats and SOCS box 3 (ASB3). ASB is a novel family of adaptor proteins bind to elongins B/C through its SOCS box and acts as an E3 ubiquitin ligase that targets proteins bound to its ankyrin repeats domain for ubiquitination and proteosomal degradation. In 293T cells, protein ubiquitination was increased by ASB3 transfection (Fig. 4a). Moreover, ubiquitinated proteins were pull down by anti-cMyc (ASB3) immunoprecipitation (Fig. 4a), suggesting that ASB3 was involved in ubiquitination. To characterize protein ubiquitination mediated by ASB3, the sandwich assay was adopted by incubating the same whole cell extracts with the PVDF-antibody array immobilized with 360 antibodies followed by anti-HA-HRP/ECL blotting analysis. Although protein ubiquitination patterns obtained from the two samples were similar, ASB3 transfection increased the intensity of the anti-HA (ubiquitin) signal for several factors including Bcl-2, Caspase14, FAF-1, Maspin, Rho C, and TNF-RII (Fig. 4b). The fact that anti-HA signal was detected at the spot immobilized with anti-cMyc antibody (Fig. 4b) strongly indicates that ASB3 was either self-ubiquinated or was associated with other proteins that were ubiquitinated. Our unpublished results indicate that by binding to TNF-RII, ASB-3 terminates TNF-RII signaling by inducing TNF-RII ubiquitination and proteosome-dependent degradation.
Identifying proteins acetylated in response to Trichostatin A treatment in HeLa cells.

Trichostatin A (TSA) is a specific inhibitor of histone deacetylase (HDAC) and can induce protein acetylation in mammalian cells. In HeLa cells, after 1 hr of treatment with TSA at indicated doses, a number of proteins including a protein with an apparent molecular mass of 54 kDa were acetylated as revealed by western blotting analysis using a polyclonal anti-acetyl lysine antibody (Fig. 5a). To evaluate transcription factor acetylation, anti-acetyl-lysine precipitates from TSA-treated HeLa cells were labeled with Alexa680 and subsequently analyzed with 12 antibodies against different STAT family members and as well as other relevant transcription factors. While STAT1, STAT2, STAT3, and STAT6 were detected as acetylated proteins in both treated and untreated cells, STAT3 acetylation was enhanced upon TSA treatment (Fig. 5b). p53 acetylation, detected in untreated HeLa cells, was slightly enhanced in the cells that received TSA treatment (Fig. 5b). When the above samples were analyzed with individual antibodies immobilized onto glass slides, p53 acetylation in response to TSA became obvious (Fig. 5c). TSA-dependent acetylation of STAT3, NF-κB p65, and tubulin were clearly detected (Fig. 5c).

To confirm the results obtained in the arrays, we chosen STAT3 and tubulin for further examination by performing immunoprecipitation and western blotting analysis. In anti-STAT3 immuno-precipitates of HeLa cells, we detected STAT3 acetylation, which was elevated by TSA treatment (Fig. 5d). TSA has been reported to induce strong α-tubulin acetylation on lysine-40 residue within the N-terminus. Utilizing a monoclonal anti-β-tubulin antibody of Santa Cruz Biotechnology Inc, we show that the strongly acetylated 54 kDa protein by TSA was tubulin (Fig. 5e). This indicates that β-tubulin may also be acetylated by TSA.

Discussion:

Antibodies conjugated to protein G- or protein A-agarose beads have long been used for precipitating proteins on an individual basis. The approach of an antibody array offers some advantages including that membranes and glass slides can be spotted with a large
number of antibodies in small volumes within a small area. The immediate advantage is that assays of this design provide identification of candidate proteins. Results presented here indicate that the intensity of the fluorescence captured by the array correlates well with the modification the protein molecule carries. Haab et al reported only 20% of the arrayed antibodies provided specific and accurate measurements of their target antigens at a concentration of 1.6 µg/ml or less. However, our results indicate that commercial antibodies, immobilized on either a PVDF membrane or a glass slide, were able to capture their target proteins from a total cell extracts. Arrays, comprising antibodies specific for various post-translational modifications, are now possible for analysis of site-specific or motif-specific modifications.

Protein samples labeled with fluorescent dye and captured by the array for analysis is a more sensitive approach as compared to sandwich assay systems. The application of fluorescent microscope or laser scanners allows one to detect samples with sufficient sensitivity to permit the analysis to be conducted with small amounts of reagents. However, this design has the potential to create signal-to-noise. Although false positive signals are inevitable for most immunoassays, the specificity of antibodies in recognizing their specific substrates becomes extremely critical for using antibody immobilization technology. For a quantitative or semi-quantitative analysis, immobilizing antibodies with equal amounts in the same orientation on two arrays will be essential for differential display of two samples. Using a high-precision contact-printing robot will allow one to print nanoliter volumes of antibodies to the array more precisely. Given that protein G or protein A preferentially interacts with Fc fragments of the antibodies, the solid support coated with protein A or G will be expected to improve antibody immobilization orientation. However, at present, the greatest challenge resides on how to normalize antigen-antibody binding affinity among various antibodies. Since different antibodies have different affinities for their specific antigens, it is difficult to make a comparison among different proteins captured by their specific antibodies even in the same array. Individual antibodies may
require precise titration to determine for their antigen peptide or protein binding intensities to overcome this problem.

In spite of the above weakness, antibody array is reliable in comparing changes of one particular protein modification under different cellular conditions. In this work, we have immobilized up to 360 antibodies, and the array can be easily expanded into a larger-scale fabrication. Antibody array will benefit undoubtedly from improved methods of array preparation, processing and analysis, and preparation of recombinant antibody libraries for generating antibodies with same affinity. Our work presented here indicates that the antibody array is a potentially powerful approach for profiling protein post-translational modification events, both known and unknown, in mammalian cells.

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References:


box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes Dev.* **12**, 3872-3881.


Legends

Fig.1. Antibodies printed on PVDF membrane and glass-slide maintain their activities to capture proteins. (a) Alexa$_{680}$-labeled whole extracts from Stat1$^{+/+}$ or Stat1$^{-/-}$ cells (0.5 mg) were incubated with PVDF-antibody arrays followed by scanning with LI-COR scanner. From 1 to 10, monoclonal or polyclonal antibodies (20ng/each) spotted on PVDF membrane included: control IgG (Cat # sc-2027), anti-STAT1 (Cat # sc-346), anti-TRADD (Cat # sc-7868), anti-c-Fos (Cat # sc-7202), anti-Caspase3 (Cat # sc-7272), anti-SHP-2 (Cat # sc-280), anti-Caspase1 (Cat # sc-622), anti-iNOS (Cat # sc-5302), anti-JAK1 (Cat # sc-277), and anti-IRF-1 (Cat # sc-497). (b) Recombinant His$_6$-STAT1 proteins labeled with Cy2 or Cy5 were visualized in a 10% SDS-PAGE gel directly with the aid of a fluorescent microscope. (c) From 1 to 7, Cy2- and Cy5-His$_6$-STAT1 were mixed at 1:0, 1:0.25, 1:0.5, 1:1, 0.5:1, 0.25:1, and 0:1 ratios. Such mixed His$_6$-STAT1 proteins (0.1 mg) were incubated with the slide-array comprising anti-His6 antibody (Cat # sc-803), anti-STAT1 antibody, and control IgG. After extensively washes, the slide-arrays were visualized with a fluorescent microscope with appropriate excitation/emission filter sets for each dye. (d) Cy2-labeled extracts of HeLa cells (0.5 mg) were incubated with the slide-array comprising 16 antibodies randomly chosen from 360 antibodies obtained from Santa Cruz Biotechnology, Inc. followed by visualization with a fluorescent microscope.

Fig.2. Characterizing multiple proteins that are tyrosine phosphorylated by EGF in A431 cells. (a) From A431 cells (5x10$^6$) treated with or without EGF (100 ng/ml) for 30 min, whole extracts were prepared as described under “Experimental Procedures” and subjected to 10% SDS-PAGE followed by western blotting using anti-pY20 antibody (Cat # sc-508). (b) Anti-pY20 immunoprecipitates were prepared from the above samples according to the procedure in “Experimental Procedures”. Alexa$_{680}$-labeled anti-pY20 precipitates were incubated with the PVDF-array comprising 25 antibodies printed in duplicates, followed by LI-COR scanning. Signal intensities apparently elevated by EGF treatment in A431 cells include EGFR (a3,4 Cat # sc-03), JAK2 (c7,8, Cat # sc-294),
STAT1 (a5,6, Cat # sc-346), STAT3 (a7,8, Cat # sc-482), STAT5a (b1,2, Cat # sc-1081), AKT1/2 (b3,4, Cat # sc-8312), and SOCS3 (e9,10, Cat # sc-9023). (c) Intensities of the signals detected in (b) were analyzed by spot densitometry on the Odyssey software version 1.1.15 and the results depict the average of the duplicates. Blue bars represent untreated and purple bars represent EGF treated. (d) Whole extracts (0.45 mg) from A431 cells treated with or without EGF were incubated with the PVDF-array comprising 25 antibodies same as above in duplicates. After extensive washes, the arrays were blotted with anti-pY20-HRP followed by ECL detection. Signals changes induced by EGF include EGFR (a3,4), STAT1 (a5,6), STAT3 (a7,8), and STAT5a (b1,2). (e) Whole extracts (0.1 mg) were prepared from A431 cells received no treatment, EGF treatment, or EGF plus Tyrophostin-AG528 (100 µM) treatment. Such prepared protein extracts were labeled with Alexa680 and analyzed with the individual antibodies immobilized as duplicates on the slide and visualized with LI-COR scanner. (f) EGFR, JAK2, and SOCS3 were immunoprecipitated from the whole extracts of A431 cells that received different treatments as indicated. These immunoprecipitates were western blotted with anti-phospho-Y1173-EGFR (Cat # sc-12351R) or with anti-pY20 as indicated. (g) Whole extracts from A431 cells with different treatments as indicated were subjected to western blotting with antibodies against STATs or against tyrosine phosphorylated STATs as indicated.

Fig.3. SODD degradation is polyubiquitination-dependent in HeLa cells. (a) Whole extracts from HeLa cells received no or TNF-α (10 ng/ml) treatment for 30 min, were subjected to western blot analysis with anti-ubiquitin antibody (Cat # sc-8017). (b) Anti-Ub immunoprecipitates (100 µg) made from HeLa cell extracts were labeled with Alexa680 according to the procedure in “Experimental Procedure”. Alexa680-labeled anti-Ub precipitates were then incubated with PVDF-array comprising 25 antibodies in duplicates, followed by scanning with LI-COR scanner. Signals enhanced by TNF-α treatment include SODD (d1,2, Cat # sc-8980), TRAF6 (e5,6, Cat # sc-8409), TRAF2 (d3,4, Cat # sc-7346), TNF-RI (c7,8, Cat # sc-8436), TNF-RII (c9,10, Cat # sc-8041), and IκBα (b3,4, Cat # sc-
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1643). (c) Intensities of the signals detected in (b) were analyzed by spot densitometry on the Odyssey software version 1.1.15 and the results depict the average of the duplicates. Blue bars represent untreated and purple bars represent TNF treated. (d) HeLa cells received no treatment, MG-132 (10 µM) treatment 2hrs, or MG-132 (10 µM) treatment 2 hrs followed by TNF-α (10 ng/ml) treatment for additional 30min. Alexa680 anti-Ub immunoprecipitates from the above HeLa cells were analyzed with individual antibodies immobilized as duplicates on slides and visualized with LI-COR scanner. (e) Whole extracts from HeLa cells in (d) were subjected to western blotting analysis with anti-Ub antibody. (f) Anti-SODD immunoprecipitates were made from HeLa cells treated as in (d). Anti-SODD immunoprecipitates were analyzed in western blot with anti-SODD antibody (upper panel) or anti-Ub antibody (lower panel).

**Fig.4. ASB3 mediates protein poly-ubiquitination in 293T cells.** (a) 293T cells were transfected with HA-Ub or HA-Ub plus cMyc-ASB3. Both whole extracts (0.5 mg) prepared from 293T transfectants and anti-c-Myc immunoprecipitates prepared from these extracts were analyzed in western blotting with anti-HA antibody. (b) Whole extracts (2 mg) of 293T cells transfected with HA-Ub or HA-Ub plus c-Myc-ASB3 were incubated with PVDF-array comprising 360 antibodies. After extensive washes, the arrays were incubated with anti-HA-HRP (Cat # sc-7392) followed by ECL detection according to “Experimental Procedures”. Signals enhanced by cMyc-ASB-3 expression include Bcl-2 (a15, Cat # sc-7382), Caspase14 (b19, Cat # sc-5628), FAF-1 (d17, Cat # sc-1885), Maspin (h8, Cat# sc-8543), Rho C (n19, Cat # sc-12116), cMyc-ASB3 (c11, Cat # sc-40), TNF-RII (l18, Cat # sc-8041), and acetyl lysine (p1, Upstate USA, Cat # 06-933).

**Fig.5. STAT3 is acetylated in response to TSA treatment in HeLa cells.** (a) HeLa cells treated with TSA at two different doses for 1hr and whole extracts from these cells were analyzed in western blotting with anti-acetyl lysine polyclonal antibody (Upstate USA, Cat # 06-933). (b) Alexa680 labeled HeLa cell extracts (0.5 mg) were incubated with PVDF-
arrays comprising antibodies against STAT1 (Cat # sc-346), STAT2 (Cat # sc-1668), STAT3 (Cat # sc-482), STAT4 (Cat # sc-486), STAT5a (Cat # sc-1081), STAT5b (Cat # sc-1656), STAT6 (Cat # sc-621), p53 (Cat # sc-126), NF-κB/p65 (Cat # sc-372), Rb (Cat # sc-102), p16INK4A (Cat # sc-759), and p21WAF1/CIP1 (Cat # sc-6246) from 1 to 12. After washes, the arrays were visualized with LI-COR scanner. (c) Alexa680 labeled anti-acetyl-lysine precipitates from the above HeLa cells were incubated with individual antibodies immobilized as duplicates on slides for 3 hrs. The slides were extensively washed and visualized with LI-COR scanner. (d) Anti-STAT3 immunoprecipitates were prepared from the whole extracts of HeLa cells received no treatment or TSA (0.2 µM) treatment for 1hr. Whole extracts in 1,2 and anti-STAT3 immunoprecipitates in 3,4 were western blotted with anti-acetyl-lysine antibody. (e) Whole extracts from HeLa cells treated with or without TSA were analyzed in western blot with polyclonal anti-acetyl-lysine antibody. After strip, the same PVDF membrane was re-blotted with monoclonal anti-β-tubulin antibody (Cat # sc-5274).
Fig. 1.

**a.**

-STAT1<sup>+/+</sup>

-STAT1<sup>−/−</sup>

**b.**

His-STAT1

**c.**

anti-His

anti-STAT1

Ctr IgG

**d.**


Fig. 3

(a) TNF: - +
IB: anti-Ub

(b) (−) (TNF)

(c) Relative Intensity

(d) TNF: - - + +
MG132: - - + +
anti-SODD-
anti-TRAF6-
anti-Ub-
Ctl-IgG-

(e) TNF: - - + +
MG132: - - + +
IP: anti-SODD
SODD-

(f) IP: anti-SODD
TNF: - - + +
MG132: - - + +
SODD-
Ub
IB: anti-Ub
Ctl-IgG-
Fig. 4.

a. 

b. 

Blot: anti-HA-HRP

IP: anti-cMyc

IB: anti-HA

- + - +

+ + + +

90 120 210

HA-Ub

cMyc-ASB3:

HA-Ub + cMyc-ASB3

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
a. IB: anti-ac-lysine 

b. -tubulin- 

IP: anti-STAT3 

TSA: - + T

1 2 3 4 5 6 7 8 9 10 11 12 

TSA (0.2μM): T

1 2 3 4 5 6 7 8 9 10 11 12 

Fig.5.