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Monitoring protein-protein-interactions in mammalian cells by Trans-SUMOylation

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Abbreviations: TRS (Trans-SUMOylation), SUMO (small ubiquitin-like modifier), Ubc9 (SUMO conjugating enzyme), HEK293 (human embryo kidney cells), p38 α (mitogen activated kinase p38 α), MK2 (mitogen activated kinase-activated protein kinase 2), MK5 (mitogen activated kinase-activated protein kinase 5), p53 (cellular tumor antigen p53), ERK3 (extracellular signal-regulated kinase 3), Y2H (yeast two hybrid), FPs (fluorescent proteins), FRET (fluorescence resonance energy transfer), PCA (protein fragment complementation assays), GFP (green fluorescent protein), UFDS (Ubc9 fusion-directed SUMOylation), HA (haemagglutinin), STAT1 (signal transducer and activator of transcription), CDK4 (cell division protein kinase 4), CRSP9 (cofactor required for Sp1 transcriptional activation, subunit 9), CSNK2B (casein kinase II subunit beta), FOS (proto-oncogene c-Fos), CDC37 (Hsp90 co-chaperone Cdc37), DRG1 (developmentally-regulated GTP-binding protein 1), HSF2BP (heat shock factor 2-binding protein), TAF10 (transcription initiation factor TFIID subunit 10), PSMC3 (Homologous-pairing protein 2 homolog), MEKK1ca (mitogen-activated protein kinase kinase kinase 1 constitutive active), TBP (TATA-box-binding protein), FKBP (peptidyl-prolyl cis-trans isomerase FKBP1A), FLAG (N-DYKDDDDK-C octapeptid), Myc (myc protooncogene), HSP27 (heat shock protein beta-1), MEK2 (dual specificity mitogen-activated protein kinase kinase 2), MEK1 (dual specificity mitogen-activated protein kinase kinase 1), ERK2 (extracellular signal-regulated kinase 2), JUN (transcription factor AP-1), Edr2 (polyhomeotic-like protein 2), HDGF (hepatoma-derived growth factor).

Synopsis

Protein-protein interactions are essential for almost all cellular processes and, hence, understanding these processes mainly depends on the identification and characterization of the relevant protein-protein interactions. Here, we introduce the concept of Trans-SUMOylation (TRS), a new method developed to identify and verify protein-protein interactions in mammalian cells *in vivo*. TRS utilizes Ubc9 fusion proteins that trans-SUMOylate coexpressed interacting proteins. Using TRS, we analysed interactions of 65 protein pairs coexpressed in HEK293 cells. We identified seven new and confirmed sixteen known protein interactions, which were determined via endogenous SUMOylation sites of the binding partners or by using SUMOylation-site-tags, respectively. Four of the new protein interactions were confirmed by GST-pull down and the p38 α /E δ r2 interaction was verified by colocalisation analysis. Functionally, this p38 α /E δ r2 interaction could possibly be involved in the recruitment of p38 α to the polycomb chromatin remodelling complex to phosphorylate Bmi1. We also used TRS to characterize protein interaction domains of the protein kinase pairs p38 α /MK2 and ERK3/MK5 and of the p38 α /p53 complex. The ability of TRS to monitor protein interactions in mammalian cells *in vivo* at levels similar to endogenous expression makes it an excellent new tool that can help for defining the protein interactome of mammalian cells.

INTRODUCTION

Specific and regulated protein-protein-interaction is essential for the existence of productive multi protein complexes that maintain and regulate almost all processes in the cell - from sugar, fat and amino acid metabolism to transcription, translation, replication, chromosome segregation and cytokinesis. Our understanding of these processes mainly depends on the identification and characterization of the specific proteins involved and on the properties of their interactions. Therefore, various methods have been developed in the past, which qualitatively and/or quantitatively analyse protein-protein interactions [reviewed in 1,2]. The most prominent methods, which have also been adapted for proteome wide analysis, are the yeast two hybrid (Y2H) system [3] and affinity purification combined with mass spectrometry [reviewed in 4]. There are several versions of both methods and each has different advantages and limitations. The isolation of endogenous protein complexes using specific antibodies for co-immunopurification could give insights into protein complexes close to the physiological reality. However, appropriate antibodies are rare and proteins weakly expressed or weak transient interactions are hardly detectable. These problems are partially solved by the use of transiently or stably overexpressed tagged proteins [5,6,7,8]. However, these may also increase the number of false positive interactors, as some protein interactions only take place during the protein extract preparation and therefore do not correspond to the actual conditions in the living cell.

Alternatively, Y2H represents a genetic approach that also identifies weak binary protein interactions and directly provides the DNA coding for the interacting proteins. It was developed for the yeast *Saccharomyces cerevisiae*, but was also adapted to *Schizosaccharomyces pombe* and to *Escherichia coli*. Y2H has been used for the identification of thousands of protein-protein-interactions of a variety of organisms [summarized in 1]. However, in these approaches proteins are expressed in a heterologous organism (yeast or *E. coli*), most of the native posttranslational modifications and chaperone protein interactions are missing and, in yeast, the system also depends on the transport of the interacting proteins to the nucleus. Accordingly, after nearly two decades of extensive use of Y2H, this method is now estimated to produce between 25 and 45% of false positive results [9]. Further *in vitro* methods for monitoring protein-protein interactions, such as pull down assays, peptide microarrays or protein co-crystallization are relatively far from physiological reality. Other methods, such as fluorescence spectroscopy and imaging technologies, attempt to analyse the interaction of proteins in their native environment. New imaging technologies coupled with genetically constructed fluorescent proteins (FPs) are used for direct co-localization of proteins by fluorescence resonance energy transfer (FRET) [10, 11] or by protein fragment complementation assays (PCA) with split-GFP [12]. These elegant methods open new opportunities to analyse single protein-protein interactions but, so far, they seem not to be suited for general and a proteome wide analysis [summarized in 13].

Here we describe Trans-SUMOylation (TRS), a new method that monitors protein-protein-interactions *in vivo* and operates under protein expression levels similar to that of the endogenous proteins. In TRS the fusion of the SUMOylating enzyme Ubc9 to the protein of interest (protein 1) results in covalent modification of an interacting protein (protein 2) by SUMOylation, a ubiquitin-like posttranslational protein modification of eukaryotic cells where a mature SUMO protein of 101 amino acids (SUMO1) is reversibly

connected to a target protein via a covalent isopeptide bond [14]. TRS is based on the recently described Ubc9 fusion-directed SUMOylation (UFDS) system [15,16] that shows strong Cis-SUMOylation of substrate proteins fused to Ubc9 (Figure 1A). When analyzing the influence of protein SUMOylation on protein-protein-interactions, we were surprised to find that Ubc9 fusion proteins can also induce the SUMOylation of binding partners in trans (Trans-SUMOylation, TRS) (Figure 1B). The analysis carried out is sufficiently detailed and universally applicable as to prove that TRS is an excellent tool for monitoring protein-protein interactions of coexpressed proteins in mammalian cells.

EXPERIMENTAL

Plasmids. The expression plasmids for HA-Ubc9, STAT1-Ubc9, p53, p53-UBC9 have been described in Jakobs et al., 2007a [15], UBC9-CDK4, CRSP9-UBC9, CSNK2B-UBC9, FOS-UBC9, GST-CSNK2B, GST-CDC37, GST-CDK4, GST-CRSP9, GST-DRG1, GST-FOS, GST-HSF2BP, GST-TAF10, GST-PSMC3, MEKK1ca, UBC9-CDC37, CSNK2B-UBC9, UBC9-CDK4, UBC9-HSF2BP, UBC9-TBP, UBC9-TAF10, UBC9-PSMC3, GST-TAF10 in Jakobs et al., 2007b [16], HA-MK5 and GST-ERK3 in Schumacher et al., 2003 [17] and the STAT1-FKBP in Zimnik et al., 2009 [18]. The expression plasmids FLAG-p38 α [19], GST-p38 α [20], Myc-MK2 [21] and HA-HSP27 [22] have been described earlier. The expression plasmids for ERK1, HA-ERK2, HA-JUN, HA-JNK2 and HA-JNK1 have been provided by Dr. Kirsten Mielke (Hannover Medical School). The expression vectors UBC9-p38 α , UBC9-MEK2, UBC9-MEK1, UBC9-ERK2, MEK1-UBC9, MEK2-UBC9, ERK2-UBC9, GST-p53, GST-ERK2, HA-CSNK2B, JUN-Ubc9, GST-Edr2 and GST-MK2 were generated by way of cloning by recombination (gateway system), the expression plasmids UBC9-MK5, UBC9-MK2, UBC9-HDGF, MK2-ST(377-393), MK2-ST(377-391), MK2-UBC9, p53K386R, p53K386R-3G-ST(377-393), p53K386R-6G-ST(377-393), p53K386R-ST(RAGP1), p53K386R-ST(377-393), p53K386R-ST(377-389), p53K386R-ST(377-391) and UCSUMO3(Q89P) by PCR amplification.

Transfection, cell lysis and western blotting. HEK293 cells were grown in Dulbecco's modified eagle's medium supplemented with fetal calf serum (10%), L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 μ g/ml). Transfection of 50-80% confluent HEK293 cells was performed in 12-well plates using polyethylenimine (1 mg/ml (in H₂O)) solution, according to Niedenthal [23]. 800ng of total transfection-purity expression vector DNA was diluted in 45 μ l of serum free DMEM. Unless stated otherwise we usually used 200ng expression plasmid for the interacting proteins and for EGFP-SUMO1 and 100ng of the expression plasmid for Ubc9 and of the empty vector pcDNA3, to reach the DNA concentration for the optimal transfection mixture. 3 μ l of the vortexed Polyethylenimine stock solution was added to the diluted DNA and mixed. After 10min incubation at room temperature, 450 μ l fresh DMEM was added to the mixture and vortexed. After removing the medium covering the cells, the transfection mix was added and the cells were incubated for 24h at 37°C at 5% CO₂. After washing the cells with 1ml PBS (room temperature) the cells were lysed in modified Laemmli (2 fold) buffer (160mM Tris-HCL, pH 6.8, 4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 0.5% (v/v) β -mercaptoethanol, 0.008% (w/v) bromphenol blue). The samples then were heated for 10min at 96°C in a heat block. For Western blot analysis the proteins were separated by SDS-PAGE, then blotted on a PVDF membrane and developed with a specific primary antibody, a HRP conjugated secondary antibody, the Immobilon® Western HRP substrate (Millipore) and the LAS-3000 imaging system (Fuji).

Protein kinase assay. 6 μ g of the purified recombinant fusion protein GST-Bmi1 expressed in *E. coli* using pDEST15-Bmi1 or the appropriate GST-Bmi1 mutant were incubated with 1.8 μ g active recombinant GST-p38 α in the absence or presence of 0,15 μ g His-MK2. As positive control, 10 μ g of recombinant Hsp25 were incubated with 0,15 μ g His-MK2 and 1,8 μ g active recombinant GST-p38 α also in a reaction mix of a final volume of 50 μ l, containing 50 mM β -glycerophosphate pH 7.4, 0.1 mM EDTA, 10 mM magnesium acetate, 0.1 mM ATP, 1.5 μ Ci of [γ -33P]ATP. After 30 min at 30 °C, reactions were terminated by adding 16 μ l of 4 SDS sample buffer. Labelling of proteins was analysed by SDS-PAGE and subsequent phospho-imaging using a Fuji Bas-1500.

Fluorescence microscopy. For subcellular localisation of GFP-tagged protein or RFP-tagged proteins the transfected cells were replated in Chambered Coverglass (Lab-Tek, Nunc) and analysed using a Leica DM IRBE microscope with the Leica TCS confocal systems program.

RESULTS

Trans-SUMOylation monitors protein-protein-interactions. SUMOylation substrate proteins are strongly SUMOylated when they are fused to the SUMO conjugating enzyme Ubc9 (Ubc9 fusion-directed SUMOylation, UFDS; Figure 1A) [15,16] and this protein fusion functionally can be replaced by a rapamycin-induced FRB/FKBP12 heterodimerization [18]. Here we have analysed whether an Ubc9 fusion protein can also SUMOylate its binding proteins (Trans-SUMOylation, TRS; Figure 1B), thus making SUMOylation a monitor for protein-protein interactions *in vivo*. Therefore, we first co-expressed a protein of interest (protein 1) fused to Ubc9 together with an interacting protein (protein 2) in HEK293 cells, which also express EGFP-SUMO1. As a result of interactions between protein 1 and 2 and subject to the condition that a SUMOylation site is available in protein 2, EGFP-SUMOylation will normally result in altered electrophoretic mobility of protein 2 and can be detected by Western blot. To analyse TRS for its ability to identify protein interactions in mammalian cells *in vivo*, we tested protein binding pairs known from the literature [summarized in the BioGRID database (www.thebiogrid.org)] and Entrez Gene [www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene]; for references also see supplementary Tables I-IV). We always co-expressed protein 2 and a protein 1-Ubc9 fusion, or, as a negative control, non-fused and non-binding protein-fused Ubc9 in the absence and presence of EGFP-tagged SUMO1. Proteins were analysed for SUMOylation by Western blot using protein 2 specific antibodies. Western blots demonstrating protein interactions detected by TRS are shown in Figure 2A-2D and in Supplementary Figure 1. In many cases intense SUMOylation of the interacting proteins can be detected indicating that TRS occurs as a general phenomenon (summarized in supplementary Tables I and II). Here it should be noted for all shown Western blots that the shift of a protein band by a SUMOylation is not simply as it is estimated by the addition of the mass of the conjugated SUMO or EGFP-SUMO. Additionally the localisation of the conjugation site at the C(N)-Terminus or in the centre of the conjugated polypeptide determines how strong the protein band is shifted. While conjugation to the ends of the polypeptide chain does not shift significantly more than due the molecular mass added, conjugation to the centre of the polypeptide chain leads often to a significant extra shift. By TRS, we confirmed sixteen established protein interactions (supplementary Table I), such as interactions between the protein kinases MK2 and p38 α (Figure 2A and 2B), between the tumor-suppressor p53 and p38 α (Figure 2C), the homodimerisation of proteasome regulatory subunit PSMC3/PSMC3 (Figure 2D), between MK2 and the polycomb member Edr2 (Supplementary Figure 1A), between the AP-1 transcription factors c-JUN and c-FOS (Supplementary Figure 1B and 1C) and also between p53 and the regulatory kinase subunit CSNK2B (Supplementary Figure 1D), (see also supplementary Table I). Furthermore, we identified seven new protein interactions (supplementary Table II): the homodimerisation of heat shock transcription factor-binding protein HSF2BP (Figure 3A), of the regulatory kinase subunit CSNK2B (Figure 3B), of the Hsp90 cochaperone CDC37 and of the protein kinase MK2 (Supplementary Figure 1E and 1F) as well as the interactions between MK2/CSNK2B (Supplementary Figure 1G), MK2/MK5 (Supplementary Figure 1G) and MK2/ERK1 (Supplementary Figure 1H). Four of the identified new protein interactions, the homodimerisation of HSF2BP (Figure 3C) and of CSNK2B (Figure 3D) as well as the interactions between MK2/MK5 (Figure 3E) and MK2/CSNK2B (Figure 3F) were confirmed by GST pull down.

We recently have shown that MK2 associates with the polycomb group complex (PcG) and regulates via its interaction with Edr2 the hematopoietic stem cell homeostasis [24]. When we verified MK2/Edr2 interactions by TRS and used p38 α and Edr2 as control, we also become aware of a positive interaction signal for p38 α and Edr2 in TRS (Supplementary Figure 1A). This novel interaction between p38 α and Edr2 could be confirmed by nuclear colocalization of both protein, when fused to GFP or RFP, respectively, and visualized in confocal microscopy (Figure 3G). Since Edr2 is part of the multi-protein PcG, we then analysed whether proteins in this complex are substrates of MK2 or p38 α . Interestingly, p38 α but not MK2 phosphorylates the Edr2-interacting PcG protein Bmi1 at the proline-directed threonine 275 (Figure 3H and 3I). Binding of p38 α to Edr2 and most likely also to MK2 leads to its recruitment to the PcG complex where it can phosphorylate the PcG protein Bmi1. The Edr2-dependent docking of p38 α to PcG and the subsequent phosphorylation of the complex component Bmi1 could indicate functional consequences and biological relevance of this novel interaction detected by TRS.

However, using EGFP-SUMO1 for eleven protein interactions described in the literature, such as STAT1/STAT1- (without tyrosine 701 phosphorylation), p53/JNK1/2- and the c-JUN/JNK2-interactions, TRS could not be detected (supplementary material, Table III; Supplementary Figure 1I-1K).

TRS using a SUMOylation-site-tag. There are several possible explanations why some protein interactions could not be confirmed by TRS: Firstly, TRS is not sensitive enough to detect very weak protein interactions, like those found between enzymes and their substrates. Secondly, the interactions identified under artificial conditions does not exist *in vivo*, thirdly, because of sterical restrictions and structural constraints in reaching the SUMOylation site by Ubc9 and, lastly, because there is no SUMOylation site present in the protein 2. To apply TRS for the latter two cases, we introduced a SUMOylation-site-tag (ST), which can introduce the ideal SUMOylation site of p53 into any protein of interest (Figure 4A). The ST(377-393)-peptide TSRHKKLMFKTEGPDSD was added to the C-terminus of the non-SUMOylated mutant p53-K386R (Figure 4A and 4B) with or without three and six glycine residues as linker, respectively (Figure 4A). As expected, the ST-fusion leads to significant increased TRS of p53K386R-ST(377-393) by Ubc9-p38 α (Figure 4C, Supplementary Figure 2A and 2D) and by CSNK2B-Ubc9 (Supplementary Figure 2D) and of MK2-ST(377-393) and MK2-ST(377-391) by Ubc9-p38 α (Supplementary Figure 2B and 2C). Furthermore, we tested other SUMOylation-site-tags (ST(377-389), ST(377-391), ST(RAGP1), ST8; Figure 4C and Supplementary Figure 2E) and demonstrated that even a peptide of 8 amino acids (ST8) functions in TRS (Figure 4C). These data indicate that STs can be utilized to extend TRS analysis to proteins which do not contain endogenous SUMOylation sites.

TRS identifies interaction domains. Next we asked whether TRS can be used to characterize protein interaction domains. To that end, we analyzed the specific interactions between the protein kinase MK5 and the atypical MAPK ERK3 (Figure 5A and 5B), which was already characterized by us in pull down experiments [17], also in TRS. First, we coexpressed Ubc9-MK5 and, as negative controls, also Ubc9-MK2 and Ubc9-p38 α together with full length GST-ERK3 and analysed the protein extracts by Western blotting (Figure 5B). The known specificity of interactions between ERK3 and MK5 [17,25] was confirmed by TRS of GST-ERK3 detected for co-expression of Ubc9-MK5 while co-expression of Ubc9-MK2 and the Ubc9-p38 α does not lead to TRS of GST-ERK3 in EGFP-SUMO1 expressing HEK293 cells. By pull down experiments of MK5 together with C-terminal ERK3-deletion mutants it has been previously shown that the overlapping ERK3-regions between amino acids 301 and 357 [17] and amino acids 330 and 340 [25] are essential for MK5 binding. Here, we analysed TRS of the C-terminal ERK3 deletion mutants as shown in Figure 5A and obtained confirmatory results with this new method: While Ubc9-MK5 leads to TRS of the ERK3(1-471) and ERK3(1-357) fragments (Figure 5B), no TRS of GST-ERK3(1-301) is observed (Figure 5B). In addition, we were able to show that there is no TRS of the entire C-terminus of ERK3(317-720) and of ERK3(101-720) (Figure 5B) by Ubc9-MK5, indicating that binding of MK5 to ERK3 requires both parts of the kinase domain of ERK3 and parts of its C-terminal extension. The analyses of MK2/p38 α , and p53/p38 α interactions by TRS also confirmed the known molecular facts (Supplementary Figure 3A-3D)

TRS of proteins expressed at endogenous levels. Protein-protein interaction analyses are prone to false positive results, particularly at the time when proteins are mixed in high concentrations or when they are co-overexpressed. To show that TRS can determine protein interactions under native *in vivo* conditions at expression levels which are similar to the endogenous ones, we analysed three different TRS pairs for protein-protein interactions at protein levels similar to endogenous ones. Therefore, the indicated amounts (330 pg - 10 ng) of specific fusion protein expression vectors were cotransfected with the indicated amounts of co-expression plasmids into HEK293 cells (Figure 6). Under these conditions and using uncleaveable (UC)SUMO3(Q89P) that reduces deconjugation we reinvestigated the TRS of GST-MK2 by p38 α -Ubc9 (Figure 6A), of GST-p38 α by MK2-Ubc9 (Figure 6B) and of GST-ERK3 by MK5-Ubc9 (Figure 6C). When the GST and Ubc9 fused proteins were expressed at levels similar to the non-fused endogenous proteins, we detected a weak TRS band (marked by empty grey arrow heads) in all lanes where the GST fusion protein was coexpressed with the Ubc9 fusion protein and SUMO (EGFP-SUMO1 and UCSUMO3). No SUMOylation was detected with the massive overexpression of Ubc9 alone (controls of Figure 6A and 6B), demonstrating the specificity of TRS. This proves that TRS can determine real *in vivo* interactions of proteins at expression levels of endogenous proteins.

DISCUSSION

The identification of protein binding partners is an essential step towards the functional characterization of a protein. Here we describe TRS, a new method to identify and study *in vivo* protein-protein interactions by coexpression in mammalian cells. TRS is based on the finding that Ubc9 fusion proteins can trans-SUMOylate their binding partners. For 16 known and 7 new protein interactions we have demonstrated that TRS can be used to monitor the binding of the proteins *in vivo* and that TRS can also contribute to

the characterization of *in vivo* interacting regions of protein pairs, such as MK5/ERK3, MK2/p38 α and p53/p38 α . While the analyses of the MK5/ERK3 complex completely confirmed the known interacting domains, we cannot exclude that the used deletions in ERK3 possibly removed the SUMOylation site(s) in that protein. This possibility was excluded in the analyses of the MK2/p38 α and p53/p38 α complex, where the deletions of protein 1 have been fused to Ubc9 and the SUMOylation substrate protein 2 remains untouched. For future analyses of interaction domains by TRS, we would always suggest to tag protein 2 with a SUMOylation-site to preserve its SUMOylatability.

Of note, using the protein pairs MK2/p38 α and MK5/ERK3, which were moderately expressed in HEK293 cells, we have demonstrated that TRS can detect the interactions of proteins at levels similar to their endogenous expression. Hence, TRS could be regarded as an *in vivo*-labelling mechanism for coexpressed proteins that interact with a specific Ubc9 fusion protein under most native conditions within the cell. The analysis we have shown here for moderately expressed proteins p38 α , MK2, MK5 and Erk3 could also be adapted to lower expressed proteins, if the protein 2 that is analysed for SUMOylation, is enriched before the Western analysis using a suitable tag.

What are the advantages and disadvantages of TRS compared to other methods for studying protein-protein interactions *in vivo* in the living cell? Often, it is suggested that co-immunoprecipitation of endogenous interacting proteins is the most suited system to confirm protein-protein interactions. Although this method starts with the native non-manipulated cellular system, immunoprecipitation of endogenous interacting proteins is performed after cell lysis in protein extracts, where proteins, which are normally separated by their distinct cellular localization or by local variations of physicochemical conditions, can artificially interact. Similar limitations exist for the methods that show protein-protein interactions by purification of protein complexes under native conditions, such as pull down, Tandem-affinity-purification (TAP) [26], the Cross-and-Capture method [27] and the Lumier-system [28] and also the QUICK-system [29], a method that can minimize unspecific contaminants, cannot reduce false-positive interactions that exclusively take place in the protein extract. TRS minimizes the probability of such false-positive results, because SUMO-labelling takes place in an interaction-dependent manner only in living cell and because the conjugating enzyme Ubc9 is directly inactivated during cell lysis by boiling in SDS loading buffer. As further control we replaced the Ubc9 fusion protein by an overexpressed unfused Ubc9. This enabled us to clearly differentiate between Ubc9-dependent and Ubc9-fusion protein-dependent SUMOylation of the target protein.

Of the genetic methods for analysis of protein-protein interactions, the most relevant are the yeast two hybrid approaches. These analyse protein-protein interactions in the living yeast cell, where various modifications of mammalian proteins necessary for the interactions cannot be generated and where yeast proteins can possibly interfere with the mammalian protein-protein interactions analysed. Therefore, mammalian two hybrid systems have been developed, which are based on the reconstitution of a transcription factor [30], of the signalling of a membrane spanning receptor signalling cascade (MAPPIT) [31], by the protein fragment complementation of an enzymatic activity (Split-TEV) [32], by the bimolecular complementation of a fluorescence signal (BIFC) [33] or also by fluorescence/bioluminescence resonance energy transfer (FRET/BRET) [34,35]. These methods may all better reflect the real situation in the cell and have been successfully used to identify interacting proteins. However, their limitations arise from the facts that interactions have to take place in a specific cellular localization (mammalian Two hybrid, MAPPIT, Split-TEV and Tango) and that there are structural constraints to reach the protein fragment complementation (Split-TEV, BIFC and FRET/BRET). Furthermore, most of these methods are necessarily combined with an indirect reporter system that can also cause false-positive results. TRS is not restricted to a special cellular localization for the protein-protein interactions and it does not need a special reporter system, because the resulting modification is detected directly at the interacting protein.

Eleven protein interactions previously described could not be confirmed by TRS using EGFP-SUMO1. Possible reasons for this could be the existence of structural constraints between the binding proteins, a problem that concerns all systems based on fusion proteins. Our observation that TRS is not efficient with all tested constructs for the p53/p38 α or the p53/ERK2 binding pairs (cf. supplementary material, Table I) supports the notion of sterical constraints of TRS. We tried to minimize these sterical constraints for TRS by the development of special linkers that will give the fused Ubc9 more of the flexibility necessary to reach the SUMOylation site of the associating proteins but so far without success (data not shown). However, we could demonstrate that both sterical constraints and missing SUMOylation sites in the protein 2 can be compensated by the introduction of highly flexible SUMOylation-site-tags (STs) into

protein 2. STs enable TRS of proteins without natural SUMOylation site(s), as shown for p53K386R, and strongly enhance the TRS of other proteins 2, such as MK2. In this context it also should be noted that the Ubc9-fusion leads to a forced SUMOylation of the fusion partner in UFDS [15,16] and also to the interaction partner in TRS (this study). Hence, even weak SUMOylation sites can display Trans-SUMOylation. However, a further explanation for missing protein 2 SUMOylation could be that some transient interactions between enzymes and substrates, such as JNKs/JUN, MK2/Hsp27 or ERK1/p53 (supplementary material, Table II), are too weak to be identified by TRS using EGFP-SUMO1. Here, further developments of TRS are under way, using SUMO with a reduced deconjugation, SUMOylation-site-tags with an increased SUMOylation or a linker-fused Ubc9 with higher conjugation activity.

TRS in its present form depends on the coexpression of the interacting proteins, where the prey protein should contain a SUMO-site or tag, and is surely suited to confirm protein interactions *in vivo* that have been identified with systems like the Y2H. Furthermore it can be used for a systematic coexpression of protein pairs to identify new interacting protein pairs *in vivo*. To ensure that as much as possible interactions will be identified, we suggest to use N- and C-terminal fusions of Ubc9 to a certain protein 1 and a protein 2 that carries a SUMOylation-site-Tag. TRS determines the protein-protein interactions of interest in the homologue organism and rules out misleading results caused by the heterologous organism used.

TRS is a completely new concept that uses a mammalian posttranslational modification system to covalently label coexpressed interacting proteins *in vivo* and is capable of studying protein-protein interactions at levels similar to endogenous expression. Therefore, TRS has the potential to contribute significantly to the physiologically validated protein-protein interactome. TRS will also be suited to monitor the action of small molecule inhibitors on protein-protein interactions in the living cell and, hence, could help to validate pharmacologically important targets. Furthermore, we have first promising indications that TRS can be the basis for the future development of a screening method for endogenous interacting proteins in mammalian cells *in vivo*, where endogenous proteins trans-SUMOylated by an expressed Ubc9 fusion protein could be purified and analysed by mass spectrometry.

AUTHOR CONTRIBUTION

Ratnesh Srivastav designed and performed experiments and analysed data. Malte Klaus, Susan Schwede and Jessica Schwermann performed experiments. Rainer Niedenthal directed and supervised the research, designed the experiments, analysed data and wrote the paper. Matthias Gaestel cosupervised the research, analysed the data and wrote the paper.

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Figure legends

Figure 1 Schematic representation of the Trans-SUMOylation (TRS) system.

(A) In the Ubc9 fusion-directed SUMOylation (UFDS), a protein directly fused to Ubc9 is SUMOylated by the SUMO loaded Ubc9 in cis. (B) A protein 2 that binds to protein 1 can be SUMOylated in trans by the SUMO loaded Ubc9 fused to protein 1.

Figure 2 Trans-SUMOylation identifies protein-protein interactions.

(A) GST-MK2 and EGFP-SUMO1 or (B) GST-p38 α and EGFP-SUMO1 were cotransfected into HEK293 cells either alone (–) or together with HA-Ubc9, p53-Ubc9, Ubc9-MK5, Ubc9-MK2, MK2-Ubc9, Ubc9-p38 α , JUN-Ubc9 or Ubc9-CSNK2B (+). (C) p53 and EGFP-SUMO1 were cotransfected into HEK293 cells either alone (–) or together with HA-Ubc9, STAT1-Ubc9, ERK2-Ubc9, Ubc9-ERK2, Ubc9-TBP, Ubc9-CDK4 or Ubc9-p38 α (+). (D) GST-PSMC3 and EGFP-SUMO1 were cotransfected into HEK293 cells either alone (–) or together with HA-Ubc9, Ubc9-PSMC3 or MK2-Ubc9 (+). 24 h after transfection the Ubc9 fusion protein binding proteins in the protein extracts of the transfectants were detected by immunoblotting using protein or tag specific antibodies (GST, p53, Ubc9). After stripping, the Ubc9 fusion proteins were detected with Ubc9 antibody (Ubc9). Binding proteins, binding proteins conjugated with coexpressed EGFP-SUMO1 (E-S1), Ubc9 fusion proteins and Ubc9 fusion proteins conjugated with coexpressed EGFP-SUMO1 (E-S1) are indicated by black arrow heads or black lines, weak Trans-SUMOylation bands are indicated by grey empty arrow heads. For all the Western blots it should be noted, that the shift of a protein band by a SUMOylation is not simply as it is estimated by the addition of the mass of the conjugated SUMO or EGFP-SUMO. Additionally the localisation of the conjugation site at the C(N)-Terminus or in the centre of the conjugated polypeptide determines strongly how much the protein band is shifted. The conjugation to the ends of the polypeptide chain does not shift strongly additional to the added mass, but the conjugation to the centre of the polypeptide chain.

Figure 3 Confirmation of protein-protein interactions identified by Trans-SUMOylation.

(A) GST-HSF2BP and EGFP-SUMO1 were cotransfected into HEK293 cells either alone (–) or together with HA-Ubc9, Ubc9-HDGF or Ubc9-HSF2BP (+). (B) GST-CSNK2B with (+) or without (–) EGFP-SUMO1 was cotransfected into HEK293 cells either alone (–) or together with CSNK2B-Ubc9 (+) and with (+) or without (–) the constitutive active (ca) MAP3-kinase MEKK1. 24 h after transfection the Ubc9 fusion protein binding proteins in the protein extracts of the transfectants were detected by immunoblotting using a GST specific antibody (GST). Binding proteins and binding proteins conjugated with coexpressed EGFP-SUMO1 (E-S1) are indicated by black arrow heads. (C) Ubc9-HSF2BP was cotransfected into HEK293 cells either with GST or together with GST-HSF2BP. (D) CSNK2B-FKBP was cotransfected into HEK293 cells either with GST or together with GST-CSNK2B. (E) GFP-MK5 was cotransfected into HEK293 cells either with GST or together with GST-MK2. (F) Ubc9-CSNK2B was cotransfected into HEK293 cells either with GST or together with GST-MK2. 24 h after transfection the GST and GST-fusion proteins bound to glutathione sepharose were pulled down from the protein extracts of the transfectants. Co-purification of Ubc9-, FKBP- and GFP-fusion proteins were detected by immunoblotting using tag specific antibodies (Ubc9, FKBP, GFP). After stripping the GST fusion proteins were detected with GST antibody (GST). The detected coexpressed proteins are indicated by black arrow heads. An unspecific band in (B) was indicated by a grey star. (G) The colocalisation of p38 α with Edr2. HEK293 cells were transfected with expression vectors for GFP-Edr2 and RFP-p38 α . Fusion proteins were visualised by fluorescence microscopy. Nuclei were stained using TO-PRO. RFP-p38 α -AGF is the fusion protein of the catalytically inactive mutant of p38 α which lacks the phosphorylatable threonine and tyrosine in the activation loop. p38 α phosphorylates Bmi1 at T275. (H) *In vitro* phosphorylation assay for Bmi1 and Hsp25 using His-MK2 alone and together with its activating MAPK GST-p38 α . Kinase assay was performed in the presence of γ -33P-ATP and phosphorylated proteins were visualised by phosphor-imaging. (I) Different potential phosphorylation site mutants corresponding to alanine replacement of the proline-directed S/T residues shown in bold in the C-terminal sequence part of Bmi1 were subjected to *in vitro* phosphorylation using GST-p38 α . Phosphorylation was detected as in H.

Figure 4 Trans-SUMOylation of SUMOylation site tags.

(A) Structure of p53 and the p53K386R mutant fused to the indicated SUMOylation-site-tags. (B) p53 and EGFP-SUMO1 or p53K386R and EGFP-SUMO1 were cotransfected into HEK293 cells either alone (–) or together with Ubc9-p38 α , Ubc9-BACH1 or MK2-Ubc9 (+). (C) Ubc9-p38 α and EGFP-SUMO1 were cotransfected into HEK293 cells together with p53wt, p53K386R, p53K386R-ST(377-393), p53K386R-3G-ST(377-393), p53K386R-6G-ST(377-393), p53K386R-ST(377-391), p53K386R-ST(377-389), p53K386R-ST(RAGP1) or p53K386R-ST8 (+). 24 h after transfection the Ubc9 fusion protein binding

proteins in the protein extracts of the transfectants were detected by immunoblotting using a p53 specific antibody). After stripping the Ubc9 fusion proteins were detected with an Ubc9 antibody (Ubc9). Binding proteins, binding proteins conjugated with coexpressed EGFP-SUMO1 (E-S1), Ubc9 fusion proteins and Ubc9 fusion proteins conjugated with coexpressed EGFP-SUMO (E-S1) are indicated by black arrow heads or black lines. An unspecific band in **C**) was indicated by a grey star.

Figure 5 Protein-protein interaction domains characterized by Trans-SUMOylation.

(**A**) Schematic representation of ERK3 deletion mutants fused to GST. (**B**) Trans-SUMOylation analysis of ERK3 deletion mutants fused to GST. GST-ERK3 and EGFP-SUMO1 were cotransfected into HEK293 cells either alone (–) or together with HA-Ubc9, Ubc9-p38 α , Ubc9-MK2 or Ubc9-MK5 (+). GST-ERK3(101-720), GST-ERK3(1-301), GST-ERK3(316-720), GST-ERK3(1-471) or GST-ERK3(1-357) were cotransfected into HEK293 cells together with HA-Ubc9 and EGFP-SUMO1 or Ubc9-MK5 and EGFP-SUMO1 (+). 24 h after transfection the Ubc9 fusion protein binding proteins in protein extracts of the transfectants were detected by immunoblotting using a GST specific antibody (GST). After stripping the Ubc9 fusion proteins were detected with an Ubc9 antibody (Ubc9). Binding proteins, binding proteins conjugated with coexpressed EGFP-SUMO1 (E-S1), Ubc9 fusion proteins and Ubc9 fusion proteins conjugated with coexpressed EGFP-SUMO (E-S1) are indicated by black arrow heads or black lines.

Figure 6 Trans-SUMOylation of proteins expressed similar to endogenous levels.

(**A**) GST-MK2 and UCSUMO3, (**B**) GST-p38 α and EGFP-SUMO1 or UCSUMO3 and (**C**) GST-ERK3 and EGFP-SUMO1 or UCSUMO3 were cotransfected into HEK293 cells either alone (–) or together with the indicated amounts of expression plasmids of HA-Ubc9, Ubc9-p38 α , MK2-Ubc9 or Ubc9-MK5 (+) to reach expression levels similar to that of endogenous proteins. 24 h after transfection the Ubc9 fusion protein binding proteins and the endogenous proteins in the protein extracts of the transfectants were separated on a 7.5% SDS-PAGE and detected by immunoblotting using protein specific antibodies (MK2, p38 α , Ubc9, ERK3, MK5). After stripping the Ubc9 fusion proteins and the endogenous proteins were detected with specific antibodies (MK2, MK5, p38 α , Ubc9). The Ubc9 and the Ubc9 fusion proteins were also detected after a separation on a 13% SDS-PAGE by immunoblotting using the Ubc9 antibody (Ubc9). The binding proteins, the binding proteins conjugated with coexpressed EGFP-SUMO1 (E-S1) or UCSUMO3 (S3), the Ubc9 fusion proteins, the HA-Ubc9, the endogenous Ubc9, MK2, MK5, ERK3 and p38 α are indicated by black arrow heads. UCSUMO3 represents the human SUMO3Q89P mutant. Weak Trans-SUMOylation bands are indicated by grey empty arrow heads. Unspecific bands in **A**) and **B**) were indicated by grey stars.

Figure 1 (Niedenthal)

Figure 2 (Niedenthal)

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Figure 4 (Niedenthal)

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Figure 6 (Niedenthal)

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