



## The SUMO-E3 ligase PIAS3 targets Pyruvate kinase M2

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Keywords:	Pyruvate kinase, PKM2, PIAS3, SUMO



## The SUMO-E3 ligase PIAS3 targets Pyruvate kinase M2

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**Abstract**

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Pyruvate kinase M2 (M2-PK) controls the rate-limiting step at the end of the glycolytic pathway in normal proliferating and tumor cells. Other functions of M2-PK in addition to its role in glycolysis are little understood. The aim of this study was to identify new cellular interaction partners of M2-PK in order to discover novel links between M2-PK and cellular functions. Here we show that the SUMO E3 ligase protein PIAS3 (inhibitor of activated STAT3) physically interacts with M2-PK and its isoenzyme M1-PK. Moreover, we demonstrate that endogenous SUMO-1-M2-PK conjugates exist in mammalian cells. Furthermore, we show that transient expression of PIAS3 but not the RING domain mutant PIAS3 (C299S, H301A) is consistent with nuclear localization of M2-PK and PIAS3 and M2-PK partially colocalize in the nucleus of these cells. This study suggests a link between PIAS3 and nuclear pyruvate kinase.

## Introduction

Pyruvate kinase (PK, ATP:pyruvate O'-phosphotransferase, EC 2.7.1.40) is one of the rate-controlling glycolytic enzymes that catalyze the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, to generate pyruvate and ATP (Staal and Rijksen, 1991). Four distinct pyruvate kinase isoenzymes (L, R, M1, and M2) occur in mammalian cells. M2-PK (alias PKM2) is the embryonic form that replaces the other isoforms in normal proliferating and in tumour cells (Mazurek et al., 2005). The human M1 and M2 isoenzymes are encoded by one gene; the specific mRNAs are generated by alternative splicing of the M1 specific exon 9 and the M2 specific exon 10, respectively (Takenaka et al., 1991). M2-PK is subject to allosteric activation by fructose-1,6-bisphosphate (FBP), an intermediate metabolite in the glycolytic pathway. Activation of M2-PK involves a shift from a dimeric to a tetrameric conformation. Tetrameric M2-PK is characterized by a high affinity to its substrate PEP and high activity at physiological PEP concentrations, whereas dimeric M2-PK has a low PEP affinity and is nearly inactive under physiological conditions (Mazurek et al., 2005). The dimer-tetramer switch of M2-PK contributes to the regulation of the glycolytic flux in proliferating cells and has been shown to be regulated by direct interaction with viral and cellular oncoproteins (Zwerschke et al., 1999; Mazurek et al., 2001), suggesting that M2-PK activity is tightly controlled in tumour cells. M2-PK is predominantly detected in many if not all human cancers (Mazurek et al., 2005) and lung cancer cells predominantly expressing M2-PK grow to large tumours in a nude mice xenografts model (Christofk et al., 2008). These data suggest that M2-PK plays an important role for tumor growth. The tetrameric form of M2-PK is associated with other glycolytic enzymes as well as with lactate dehydrogenase, nucleotide diphosphate kinase, and adenylate kinase, within a protein complex, referred to as the glycolytic enzyme complex (Mazurek et al., 1996; Mazurek et al., 2001). In contrast, dimeric and also a monomeric form of M2-PK are not associated with the glycolytic complex (Mazurek et al., 1996). Monomeric M2-PK was described as a cytosolic thyroid hormone binding protein (Kato et al., 1989), and, recently nuclear localization and a role in

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3 transcriptional regulation was described for M2-PK (Lee et al., 2008); however, non-glycolytic  
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5 functions of M2-PK are little understood.  
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10 SUMO, a small ubiquitin-like modifier protein that is highly conserved from yeast to human, is  
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12 conjugated to a large number of cellular target proteins (Uzunova et al., 2007). Similar to  
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14 enzymatic ubiquitination, the conjugation of specific SUMO proteins (SUMO-1-3) to target  
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16 proteins requires an E1-activating enzyme (Aos1/Uba2) as well as an E2-type SUMO-1-  
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18 conjugating enzyme (Ubc9; Johnson, 2004). More recently, proteins that increase the  
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20 efficiency of SUMO conjugation have been discovered. The members of the mammalian  
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22 PIAS (Protein Inhibitor of Activated STAT) protein family, originally identified as cytokine-  
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24 induced inhibitors of the STAT family of transcription factors (Chung et al., 1997), belong to  
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26 this protein class, referred to as SUMO E3 ligases. The PIAS family consists of seven  
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28 structurally related proteins (PIAS1, PIAS3, PIAS3 $\beta$ , PIASx $\alpha$ , PIASx $\beta$ , PIASy and PIASyE6)  
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30 (Shuai and Liu, 2005). Similar to many ubiquitin E3 ligases, these proteins contain a  
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32 predicted RING-finger-like structure which has been shown to be essential for their SUMO  
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34 E3 ligase activities toward various target proteins. In contrast to the well-known function of  
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36 polyubiquitin conjugation in proteasome-mediated protein degradation, sumoylation seems to  
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38 be rarely involved in protein degradation processes. There is evidence that SUMO-2/3  
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40 conjugates in human cells are controlled via degradation by the proteasome while SUMO-1  
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42 conjugates show little changes upon inhibition of the proteasome (Uzunova et al., 2007).  
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44 Sumoylation is a highly dynamic process and its outcomes are highly diverse, ranging from  
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46 changes in subcellular localization, transcriptional regulation to altered activity and stability of  
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48 the modified protein (Johnson, 2004). PIAS proteins do not operate merely as SUMO E3s,  
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50 since their co-regulator effects are often independent of their RING finger but dependent on  
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52 their SIM (SUMO-interacting motif) or SAP (scaffold attachment factor-A/B/acinus/PIAS)  
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54 domain (Palvimo, 2007). The modulator activity of the PIAS/SUMO system has so far mainly  
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56 been shown to involve altered subnuclear targeting and/or assembly of transcription  
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5 The aim of this study was to discover novel cellular M2-PK-binding proteins. We identified  
6 the SUMO E3 ligase PIAS3 (Chung et al., 1997) as a novel M2-PK-interacting protein and  
7 analyzed functional consequences of this interaction.  
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For Peer Review

## Materials and Methods

### *Interaction analysis in yeast*

LexA-M2-PK was used as bait in the yeast two-hybrid screen to identify cDNAs for M2-PK-binding proteins from a human WI38 fibroblast cDNA expression library (pB42-WI38 cDNA::TRP1) (Zwerschke et al., 1999). The pB42-WI38 cDNA::TRP1 plasmids express the B42 transactivation domain-prey fusions proteins from the inducible GAL1 promoter. Yeast strain EGY48/pSH1834 (MATa, his3, ura3, trp1, leu2::lexA<sub>o6</sub>-pLEU2/LexA<sub>o8</sub>-GAL1-lacZ::URA3) was used for both the LEU2 and  $\beta$ -galactosidase reporter gene assays. For determination of reporter gene activity, EGY48/pSH1834 was transformed with pEG202::HIS3 plasmids expressing the LexA-M2-PK, LexA-M1-PK or LexA-APP<sub>599-695</sub> fusion protein, together with the plasmid pB42-HA-PIAS3<sub>383-619</sub>::TRP1, and it was selected for leucine prototrophy as previously described (Zwerschke et al., 1999). Alternatively,  $\beta$ -galactosidase activity was determined in cellular extracts as previously described (Zwerschke et al., 1996). The various LexA fusion proteins were expressed to the same level, as confirmed by direct immunoblotting, using a polyclonal antibody against LexA.

### *Plasmids*

The complete human M2-PK cDNA was isolated by EcoRI/XhoI restriction digest from the yeast prey vector pB42-M2-PK::TRP1 (Zwerschke et al., 1999) and inserted in frame to GST in the bacterial expression vector pGEX-4T-1 (Amersham Biosciences, Vienna, Austria) to generate pGEX-4T-1/M2-PK, and fused in frame to LexA in the yeast two-hybrid bait vector pEG202 (Zwerschke et al., 1999) to generate the bait vector pEG202/M2-PK. To construct the bait vector pEG202/APP<sub>599-695</sub>, the human APP cDNA encoding for the amino acids 599-695 was amplified by PCR and inserted as an EcoRI/NotI fragment in frame to LexA into pEG202. To generate pGEX-4T-1/M1-PK and pEG202/M1-PK the sequence encoding the M2-PK specific region was exchanged with the M1-PK specific region isolated as SphI/XhoI fragment from the vector pX/M1-PK (Mazurek et al., 1996). The yeast expression vector

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3 pEG202/M2-PK<sub>1-348</sub> encoding for the carboxyl-terminal deletion mutant of M2-PK from amino  
4 acid residues 1-348 fused to the B42 transactivation domain was constructed by the deletion  
5 of a MscI/XhoI fragment in pEG202/M2-PK spanning from the 5' MscI side to the stop codon  
6 of M2-PK. The human PIAS3 cDNA was amplified from the RZPD clone  
7 IDIMAGp958J08198Q2 (Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin,  
8 Germany) by PCR and ligated as EcoRI/EcoRI fragment into the EcoRI site of the pEG202,  
9 pGEX-4T-1 and pUHD10-3 to generate pEG202/PIAS3, pGEX-4T-1/PIAS3 and pUHD10-  
10 3/PIAS3 respectively. All plasmids were confirmed by sequencing. The plasmids Flag-  
11 mPIAS3 wild type and Flag-mPIAS3 (C299S, H301A) are gifts of Fang Liu (see, Long et al.,  
12 2004).  
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#### 27 *In vitro interaction analysis*

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29 GST pull down assays were performed essentially as described (Mannhardt et al., 2000),  
30 using 5 µg purified GST, GST-M2-PK or GST-PIAS3 proteins and purified PIAS3 protein or  
31 U-2 OS cell lysates. PIAS3 protein was purified by immunoprecipitation using anti-PIAS3  
32 antibodies (AP,1245a, Abgent, Heidelberg, Germany) and protein A and G agarose beads.  
33 The beads were washed and the purified PIAS3 protein was eluted using 0.1 M glycine HCl,  
34 pH 3.5, into vials containing 1 M Tris, pH 8.0. The purified PIAS3 protein (<200ng) was either  
35 incubated with purified GST-M2-PK (800 ng) or GST (1000 ng).  
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#### 47 *Coimmunoprecipitation experiments*

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49 Co-immunoprecipitation experiments were performed essentially as described (Mannhardt et  
50 al., 2000).  
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#### 55 *Western blotting experiments*

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57 Immunoblot analysis was performed essentially as described (Mannhardt et al., 2000) using  
58 antibodies against LexA (sc-7544, IgG1, mouse monoclonal Santa Cruz Biotechnology,  
59 Vienna, Austria), M2-PK (clone DF4, mouse monoclonal, ScheBo Biotech, Wetttenberg,  
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3 Germany), SUMO-1 (33-2400, mouse monoclonal, Zymed, Vienna, Austria, or A-712, rabbit  
4 polyclonal, BostonBiochem, USA), M1/2-PK (200-1178, goat polyclonal, Rockland, Vienna,  
5 Austria), HA1 (sc-805, rabbit polyclonal, Santa Cruz Biotechnology) and PIAS3 (AP,1245a,  
6 rabbit polyclonal, Abgent). Further antibodies used: anti-rabbit, W401B, IgG, HRP conjugate;  
7 anti-mouse W4021, IgG, HRP conjugate; anti-goat, W4011, IgG, HRP conjugate (Promega,  
8 Vienna, Austria). Anti-goat IgG (FITC-conjugate Jackson, Vienna, Austria). Anti-rabbit IgG,  
9 TRITC-conjugate (DAKO, Vienna, Austria).

#### 10 11 12 *In vivo sumoylation assay*

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14 To prevent desumoylation of the target proteins, U-2 OS cells were resuspended in SUMO  
15 immunoprecipitation buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2% SDS) and incubated  
16 at 95°C for 10 minutes to inactivate desumoylating enzymes (Buschmann et al., 2001). The  
17 lysate was diluted 1:10 in dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton-  
18 X-100) and centrifuged at 20,000g and 4°C. After preclearing with A/G agarose beads (50  
19 µg/1 mg lysate) M2-PK was precipitated over night with goat polyclonal antibodies against  
20 pyruvate kinase (Rockland Immunochemicals, Gilbertsville, PA) and A/G agarose beads. The  
21 precipitates were washed with dilution buffer, separated by SDS-PAGE and blotted onto a  
22 PVDF membrane. The PVDF membrane was probed with monoclonal anti-M2-PK or  
23 monoclonal anti-SUMO-1 antibodies.

#### 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 *Cell culture and transfection*

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49 The human osteosarcoma cell line U-2 OS was cultured in DMEM plus 10% fetal calf serum  
50 as described (Mannhardt et al., 2000) and the mouse NIH3T3 cell line was cultured in DMEM  
51 plus 10% calf serum as described (Zwerschke et al., 1999). Transient transfection of the  
52 expression vector pUHD-3/PIAS3 was performed using Effectene (Qiagen, Hilden,  
53 Germany).

#### 54 55 56 57 58 59 60 *Indirect immunofluorescence analysis*

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3 Indirect immunofluorescence analysis was performed essentially as described previously  
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5 (Mannhardt et al., 2000). Briefly, cells were grown to about 80% confluence on glass cover  
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7 slips and transiently transfected (Effectene, Qiagen, Germany). At 30 h post transfection,  
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9 cells were prepared for indirect immunofluorescence according to standard protocols,  
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11 including fixation with 4%PFA/0.2% Triton-X-100 and incubation with primary antibodies ( $\alpha$ -  
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13 PIAS3 antibody, Abgent, clone AP1245a, Vienna;  $\alpha$ -M2-PK antibody, Rockland, Vienna) and  
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15 TRITC- or FITC-conjugated secondary antibodies. Nuclear staining was conducted with TO-  
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17 PRO-3 (Molecular Probes, Göttingen, Germany). Samples were viewed by indirect  
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19 immunofluorescence microscopy using the confocal scanning system MicroRadiance (Bio-  
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21 Rad, Hempstead, U.K.) in combination with a Zeiss Axiophot microscope. The following  
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23 filters were used for FITC-derived and TRITC-derived fluorescence: excitation for FITC 488  
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25 nm, TRITC 543 nm and emission for FITC 515 to 530 nm and TRITC >570 nm.  
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## Results

### Identification of PIAS3 as M2-PK binding protein

To better understand the function of M2-PK, a yeast two-hybrid screen for cellular M2-PK-binding proteins was conducted. We used full length M2-PK fused in frame to the LexA-DNA-binding domain as bait (Zwerschke et al., 1999). LexA-M2-PK can bind to the LexA binding sites of a synthetic LEU2 reporter gene, and thereby allows monitoring of the interaction between M2-PK and a second hybrid protein containing the B42 transactivation domain fused in-frame to a heterologous cDNA. When a galactose inducible human fibroblast cDNA expression library (pB42-WI38 cDNA::TRP1) was coexpressed with LexA-M2-PK in the yeast strain EGY48/pSH1834 and screened for transformants that grew on leucine-deficient medium, cDNAs encoding M2-PK and fragments thereof were repeatedly isolated (G. Spoden and W. Zwerschke, unpublished findings), reflecting the tendency for M2-PK to form tetramers and dimers (Zwerschke et al., 1999). Intriguingly, the M2-PK bait identified a cDNA encoding for the intact carboxyl-terminal substrate binding domain (amino acids 383-619) of protein inhibitor of activated STAT3 (PIAS3) (Chung et al., 1997), which was more recently identified as a SUMO E3 ligase involved in SUMO-1 modification and transcriptional repression of interferon regulatory factor-1 (Nakagawa and Yokosawa, 2002) and microphthalmia transcription factor (Miller et al., 2005). Coexpression of the PIAS3<sub>383-619</sub>-HA1-B42 fusion protein with LexA-M2-PK (Fig. 1A) enabled the yeast strain EGY48/pSH1834 to grow on galactose but not glucose minimal plates, indicating that leucine prototrophy depends on expression of the M2-PK-B42 fusion protein (Fig. 1B). PIAS3 also bound to the M1-PK isoenzyme in this assay (Fig. 1B), suggesting that the M2-PK specific region is not necessary for the pyruvate kinase/PIAS3 interaction. The inability of the unrelated fusion protein LexA-APP<sub>599-695</sub> to bind to B42-HA-PIAS3<sub>383-619</sub> indicates that the M-type pyruvate kinase sequences are essential for binding to PIAS3. These findings were corroborated using lacZ as a second reporter gene. High  $\beta$ -galactosidase activity (blue color)

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3 was observed when B42-HA-PIAS3<sub>383-619</sub> was coexpressed with either LexA-M2-PK or LexA-  
4 M1-PK, but not with the unrelated fusion protein LexA-APP<sub>599-695</sub> (Fig. 1B, bottom panel).  
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10 To analyze whether M2-PK also interacts with the PIAS3 full-length protein, purified GST-  
11 M2-PK was incubated with extracts from human U-2 OS cells overexpressing wild type  
12 PIAS3. PIAS3 specifically bound to GST-M2-PK in this assay, whereas no binding was  
13 observed for GST (Fig. 2A, left). Moreover, the incubation of GST-PIAS3 full-length protein  
14 with lysates from human U-2 OS cells demonstrates that human M2-PK binds specifically to  
15 purified GST-PIAS3, but not to GST alone (Fig. 2A, right). These data suggest that M2-PK  
16 interacts with PIAS3 in vitro. To map the PIAS3-interacting region on M2-PK, we incubated  
17 GST-PIAS3 either with the M2-PK full-length protein (aa 1-531) or the carboxyl-terminal  
18 deletion mutant M2-PK<sub>1-348</sub> lacking the M2-PK specific region (Fig. 2B, left). Both proteins  
19 were expressed in yeast cells and the lysates incubated with purified GST-PIAS3 and GST.  
20 We found that M2-PK<sub>1-348</sub> bound as strongly as the M2-PK full-length protein to GST-PIAS3  
21 (Fig. 2B, left). This underlines the specificity of the PIAS3/M2-PK protein-protein interaction  
22 and suggests that M2-PK<sub>1-348</sub> is sufficient for PIAS3 binding. This is in keeping with the  
23 finding that the B42-HA-PIAS3<sub>383-619</sub> protein specifically interacts with both M-type pyruvate  
24 kinase isoenzymes (Fig. 1), whose amino acid sequences are identical in this region. The  
25 interaction of M2-PK with the intact carboxyl-terminal substrate binding domain of the PIAS3  
26 protein found in living yeast cells (Fig. 1) was corroborated in a GST pull down experiment.  
27 GST-M2-PK bound specifically and with high affinity to B42-HA-PIAS3<sub>383-619</sub> (Fig. 2B, right),  
28 underlining that it is the C-terminal domain of PIAS3 that interacts with M2-PK. Collectively,  
29 these results map the interaction domains to the amino-terminal part of M2-PK (residues 1-  
30 348) and the carboxyl-terminal domain of PIAS3 (residues 383-619).  
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57 To show that the described interaction also occurs in cell extracts, co-immunoprecipitation  
58 experiments were conducted using human U-2 OS cells transiently transfected with a CMV  
59 promoter driven PIAS3 expression vector (Fig. 3). Endogenous M2-PK was specifically co-  
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3 precipitated with PIAS3 using anti-PIAS3 antibodies, but not with an unrelated polyclonal  
4 control serum (Fig. 3, left). M2-PK antibodies precipitated M2-PK from U-2 OS extracts and  
5 co-immunoprecipitation of PIAS3 was observed, whereas an unrelated polyclonal control  
6 serum precipitated neither M2-PK nor PIAS3 (Fig. 3, right). These findings suggest that M2-  
7 PK can form a complex with the SUMO E3 ligase PIAS3 in extracts from human cells.  
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### 16 **A subfraction of M2-PK is SUMO-1-conjugated**

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18 Computer-aided analysis of the human M2-PK sequence revealed eight putative SUMO-1  
19 acceptor sequences in the M2-PK molecule (supplementary Fig. 1), suggesting that it may  
20 be a sumoylation target. To examine whether endogenous M2-PK is covalently conjugated  
21 with SUMO-1, U-2 OS cell extracts were prepared in SDS-containing lysate buffer to avoid  
22 desumoylation, and M2-PK was immunoprecipitated from the extracts overnight. The  
23 precipitates were analyzed by Western blotting with antibodies against M2-PK and SUMO-1,  
24 respectively (Fig. 4A). The anti-M2-PK antibody recognized a specific band at 58 kD,  
25 corresponding to unmodified M2-PK, and two slower migrating bands at approximately 80  
26 and 110 kD (Fig. 4A, left, lane 2). The anti-SUMO-1 antibody recognized the two slower  
27 migrating bands at 80 and 110 kD (Fig. 4A, right, lane 2). The 110 kD protein was also  
28 immunoprecipitated by isotypic control antibodies (Fig. 4A, right, lane 1), indicating that only  
29 the 58 and 85 kD bands are specifically immunoprecipitated by the anti-M2-PK antibodies.  
30 This data suggests that the 80 kD band is consistent with the conjugation of SUMO-1 to  
31 endogenous M2-PK. Most of the protein recognized by the anti-M2-PK antibodies runs at 58  
32 kD (Fig. 4A), suggesting that only a part of the endogenous M2-PK exists as SUMO-1  
33 conjugate and/or that the M2-PK-SUMO-1 conjugates are relatively unstable. To address this  
34 question, the U-2 OS cells were harvested in a stabilizing SDS-containing lysis buffer and  
35 either immediately processed for Western blotting (Fig. 4B, lane 1) or incubated for six (Fig.  
36 4B, lane 2) or eighteen (Fig. 4B, lane 3) hours at 4°C before processing. Over time the  
37 intensity of the high molecular weight bands recognized by the anti-M2-PK antibody was  
38 reduced (Fig. 4B, compare lanes 1, 2 and 3) - especially after eighteen hours, the incubation  
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3 time used for the immunoprecipitation experiment in Fig. 4A. These data suggest that higher  
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5 molecular weight M2-PK conjugates exist, but that these proteins are relatively unstable,  
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7 explaining the weak signals obtained in the Co-IP (Fig. 4A).  
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11 To analyze whether PIAS3 specifically enhances M2-PK sumoylation, PIAS3 was transiently  
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13 expressed in U-2 OS cells (Fig. 4C) and M2-PK was immunoprecipitated from PIAS3-  
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15 overexpressing U-2 OS cells. When the immunoprecipitates were probed with anti-M2-PK  
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17 (Fig. 4A, left, lane 3) and anti-SUMO-1 (Fig. 4A, right, lane 3) antibodies, it was found that  
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19 the intensity of the 80 kD M2-PK-SUMO-1 conjugate was slightly increased in the PIAS3 co-  
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21 expressing cells. The triple repetition of this immunoprecipitation experiment and  
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23 quantification of the intensity of the 80 kD M2-PK-SUMO-1 band from PIAS3 non-  
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25 overexpressing and PIAS3 overexpressing U-2 OS cells by densitometry showed that PIAS3  
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27 produced a slight increase in M2-PK sumoylation (Fig. 4D).  
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### 33 **Expression of PIAS3 coincides with nuclear localization of M2-PK**

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35 Since there is precedence that sumoylation can influence the subcellular localization of target  
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37 proteins acting in carbohydrate metabolism, as has been shown for the glucose transporter  
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39 GLUT4 and GLUT1 (Giorgino et al., 2000; Lalioti et al., 2002), we investigated the subcellular  
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41 localization of M2-PK in PIAS3 overexpressing cells. To this end, we transiently transfected a  
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43 PIAS3 expression vector into U-2 OS cells. Costaining of PIAS3 with anti-PIAS3 antibodies  
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45 and the DNA stain TO-PRO-3 revealed that PIAS3 (green) was predominantly localized in  
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47 the nucleus (red), a nuclear speckled staining for PIAS3 was found, as monitored by indirect  
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49 immunofluorescence experiments using a confocal laser-scanning microscope (Fig. 5A).  
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51 Costaining of M2-PK and TO-PRO-3 revealed that M2-PK (green) was also localized in the  
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53 nucleus (red) in PIAS3 overexpressing cells (Fig. 5B); however, not in U-2 OS cells  
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55 containing only empty vector (Fig. 5C). To analyze whether M2PK and PIAS3 colocalize in  
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57 the nucleus of PIAS3 overexpressing U-2 OS cells, we conducted a costaining with anti-M2-  
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59 PK and anti-PIAS3 antibodies and visualized the staining by either red (M2-PK) or green  
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3 (PIAS3) fluorescence (Fig. 5D). U-2 OS cells transiently expressing PIAS3 (Fig. 5D, middle,  
4 green) showed a strong nuclear staining signal for M2-PK, although significant levels of M2-  
5 PK were still detectable in the cytoplasm (Fig. 5D, left). This is consistent with the finding that  
6 only a part of endogenous M2-PK is SUMO-1 conjugated (Fig. 4). In the PIAS3  
7 overexpressing cells, M2-PK was distributed throughout the nucleus, whereas PIAS3 was  
8 predominantly detected in defined nuclear speckles, and colocalization between M2-PK and  
9 PIAS3 was observed predominantly at these nuclear structures (Fig. 5D, right, yellow). In  
10 mock-transfected U-2 OS cells, the anti-M2-PK antibodies stained only structures in the  
11 cytoplasm but not in the nucleus, and endogenous PIAS3 was not detectable (Fig. 5E). No  
12 staining of U-2 OS cells was observed when only the secondary anti-rabbit and anti-goat  
13 IgGs were added (Fig. 5F). To corroborate these findings, PIAS3 was transiently expressed  
14 in NIH3T3 mouse fibroblasts. Similar to our findings in U-2 OS cells, ectopic expression of  
15 PIAS3 resulted in colocalization of PIAS3 with M2-PK in the nucleus of NIH 3T3 cells (Fig.  
16 5G). Together these experiments demonstrate that PIAS3 and M2-PK specifically co-localize  
17 in the nucleus in PIAS3 overexpressing cells.

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36 To test whether the increased nuclear localization of M2-PK depends on the catalytically  
37 active RING domain in PIAS3, we employed the catalytically inactive PIAS3 RING domain  
38 mutant Flag-mPIAS3 (C299S, H301A) described in Long et al., (2004), and investigated the  
39 impact of the Flag-mPIAS3 RING domain mutant and of Flag-mPIAS3 wild type on nuclear  
40 localization of M2-PK in U2-OS cells (Fig.6). We transiently expressed both proteins in U2-  
41 OS cells and detected nuclear M2-PK only in the Flag-mPIAS3 (wild type) (Fig. 6B) but not in  
42 the Flag-mPIAS3 (C299S, H301A) overexpressing cells (Fig.6C). For this reason, we  
43 conclude that the intact RING domain in PIAS3 is important for nuclear localization of M2-PK.  
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## Discussion

In this study we have identified the SUMO E3 ligase PIAS3 (Chung et al., 1997) as interaction partner of the glycolytic isoenzymes M2-PK and M1-PK in a two-hybrid screen. The interaction between M2-PK and PIAS3 was further corroborated in GST-pull down assays and by co-immunoprecipitation of PIAS3 and M2-PK from extracts of human U-2 OS cells. Moreover, we found that a substantial fraction of endogenous M2-PK is covalently conjugated to SUMO-1 and we present data that M2-PK colocalizes with PIAS3 in nuclear structures in cells transiently overexpressing PIAS3. These data suggest a functional link between PIAS3 and M2-PK.

### M2-PK as sumoylation substrate

Since SUMO-1 has an apparent molecular mass of approximately 20 kD (Matunis et al., 1996) and the monosumoylation of RanGAP1 increases its apparent MW by roughly 25 kD (Matunis et al., 1996), our identification of an additional M2PK band with an apparent MW of 80 kD in Fig. 4, which is also recognized by SUMO-1 antibodies, is consistent with the addition of one SUMO-1 per M2-PK. Using the SUMO plot program ([www.abgent.com/doc/sumoplot](http://www.abgent.com/doc/sumoplot)), we identified eight potential sumoylation motifs (YKXD/E; Y bulky amino acid, K attachment site lysine, X any amino acid) in M2-PK (supplementary Fig. 1). Moreover, analysis of the X-ray structure of human M2-PK (Dombrauckas et al., 2005) showed that all eight lysine residues are exposed on the surface of the enzyme. This underlines our finding that M2-PK is sumoylated. The identification of the sumoylated lysine residue(s) in M2-PK requires further study.

The identification of human pyruvate kinase as target for SUMO-1 conjugation in this study is reminiscent of similar findings in yeast. Thus, the *S.cerevisiae* homolog of pyruvate kinase Pyk1/Cdc19 has been identified as a SUMO substrate in mass spectrometry-based proteome-wide approaches (Hannich et al. 2004, Zhou et al. 2004, Panse et al. 2004, Wohlschlegel et al., 2004, Denison et al. 2005), suggesting that sumoylation of pyruvate kinase, as described here, represents an evolutionarily conserved process.



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3 The two human M-type pyruvate kinase isoenzymes consist of 531 amino acid residues and  
4 differ only in 22 amino acid residues in a 45 residues-long domain encoded by the respective  
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The two human M-type pyruvate kinase isoenzymes consist of 531 amino acid residues and differ only in 22 amino acid residues in a 45 residues-long domain encoded by the respective exons (Dombrauckas et al., 2005). The type-specific domains are important for the interaction of the M1 and M2 pyruvate kinase subunits, respectively, and although structurally very similar (Wooll et al., 2001; Dombrauckas et al., 2005), the non-allosteric M1 isoenzyme exists only as tetramer, while M2-PK is an allosteric enzyme that can exist in different conformations. All hypothetical sumoylation motifs are localized in domains common to M1- and M2-PK (supplementary Fig. 1), suggesting that M1-PK may also be subject to sumoylation. This is in keeping with our finding that PIAS3 specifically binds to both isoenzymes, M2-PK and M1-PK in a yeast two-hybrid assay (Fig. 1), and further underlined by our finding that the amino-terminal domain, which is common to both M-type isoenzymes, is sufficient for the PIAS3/pyruvate kinase interaction (Fig. 2). While these findings suggest that PIAS3 interacts with both M1-PK and M2-PK subunits, one cannot rule out that the quaternary structure of pyruvate kinase influences the strength of the M2-PK/PIAS3 interaction. It is conceivable that also the other two pyruvate kinase isoenzymes, L-PK and R-PK, can exist as sumoylated proteins in cells expressing these PK isoforms. This question needs, however, further studies.

### **M2-PK as PIAS3 target protein**

We found that overexpression of PIAS3 induces a moderate increase in the sumoylation of M2-PK. This is in line with the finding that only a fraction of the endogenous M2-PK was detected in a complex with PIAS3 in the co-immunoprecipitation experiments and modified by SUMO. These results suggest that PIAS3 enhances sumoylation of M2-PK, but that the M2-PK/PIAS3 interaction occurs between a rather small fraction of the two proteins. However, the SUMO E2 enzyme Ubc9 can directly bind the consensus sequence YKXD/E in a given substrate and Ubc9 together with the SUMO E1 enzyme are sufficient to efficiently sumoylate substrates *in vitro* in the absence of an E3 (Johnson, 2004; Reverter and Lima, 2005), indicating that E3 enzymes can enhance sumoylation without being indispensable.

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3 Moreover, there are examples in which the effect of PIAS proteins on substrate function is  
4 independent of their SUMO ligase activity (their RING finger), suggesting that the role of  
5 PIAS proteins extends beyond modifying the outputs of the SUMO pathway (Johnson, 2004;  
6 Palvimo, 2007). Thus, the interaction between PIAS3 and M2PK, as shown here, could  
7 reflect either an enzyme-substrate complex with a short half life or another type of regulatory  
8 interaction. In fact, only a few proteins are quantitatively sumoylated. Instead, most targets  
9 appear to be modified to a small percentage at steady state (usually less than 5%) (Geiss-  
10 Friedlander and Melchior, 2007). It is important to consider that targets can undergo rapid  
11 cycles of modification and demodification. Although the equilibrium might lie on the side of  
12 the unmodified form, the whole pool of a given protein might be affected by sumoylation in a  
13 short window of time and there is precedence that low-level sumoylation can cause large  
14 effects (Geiss-Friedlander and Melchior, 2007). When endogenous PIAS3 was partially  
15 depleted by siRNA-mediated gene knockdown, no discernable differences in the degree of  
16 M2-PK were observed (data not shown). When the catalytically inactive mouse PIAS3 RING  
17 domain mutant PIAS3 (C299S, H301A) (Long et al., 2004) was expressed in U2-OS cells  
18 instead of wild type PIAS3, no nuclear localization of M2-PK was observed (Fig. 6). Thus,  
19 while the role of PIAS3 for sumoylation of M2-PK remains to be defined, our data suggest  
20 that PIAS3 specifically induces nuclear localization of M2-PK and this effect depends on the  
21 RING domain of PIAS3.

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44 The interaction of M2-PK with PIAS3 involves the intact carboxyl-terminal region of PIAS3  
45 from amino acid residues 383 to 619. Although the approximately 400 residues-long  
46 conserved amino-terminal region of PIAS3 is well characterized - it contains the RING finger-  
47 like zinc-binding domain mediating the SUMO-E3-ligase activity of PIAS proteins (Kotaja et  
48 al., 2002) and binds directly to Ubc9 (Kahyo et al., 2001) - the exact functions of the less  
49 conserved carboxyl-terminal region remain to be defined. This region contains an acidic  
50 motif, a serine/threonine rich region, and is involved in the interaction with specific substrate  
51 proteins (Shuai and Liu, 2005), as has been shown for some transcriptional regulators  
52 (Nakagawa and Yokosawa, 2002; Jimenez-Lara et al., 2001; Tirard et al., 2004). Although  
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3 PIAS proteins are localized predominantly in the nucleus and transcription is a major process  
4 regulated by these SUMO E3 ligases, additional processes are regulated by sumoylation and  
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7 PIAS proteins have been shown to interact with various cellular proteins, for instance  
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10 enzymes involved in DNA-replication or proteins of the cytoplasmic septin family of  
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12 cytoskeletal proteins (Johnson, 2004). Whereas SUMO conjugation is important for the  
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14 targeting of glucose transporters (GLUT4 and GLUT1) from intracellular sites to the  
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16 cytoplasmic membrane (Giorgino et al., 2000; Lalioti et al., 2002), the direct interaction of a  
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18 SUMO E3 ligase with a glycolytic enzyme, along with its subcellular relocalization, has not  
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20 previously been shown.  
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25 In this study it is shown, that overexpression of PIAS3 leads to nuclear localization of M2-PK  
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27 and the colocalization of this enzyme with PIAS3 in the nucleus of different cell types.  
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30 Little is known about M2-PK beside its important metabolic function as glycolytic enzyme in  
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32 the cytosol (reviewed in Mazurek et al., 2005). A monomeric form of M2-PK with low  
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34 enzymatic activity was previously described as a cytosolic thyroid hormone-binding protein  
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36 (Kato et al., 1989), and a subsequent study suggested that monomeric M2-PK has a role in  
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38 the regulation of thyroid hormone receptor-dependent transcription (Ashizawa, and Cheng,  
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40 1992). Previous work also provided evidence for DNA-binding and histone H1 kinase activity  
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42 of M2-PK (Guminska et al., 1988), and recent work demonstrated that M2PK can exist as a  
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44 nuclear protein. Thus, interleukin-3 induces nuclear translocation of M2-PK and this  
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46 enhances cell proliferation (Hoshino et al., 2007). Another study showed that M2-PK  
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48 translocates into the nucleus after apoptotic stimuli and contributes to the induction of  
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50 programmed cell death (Steták et al, 2007). Furthermore, a role in transcriptional regulation  
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52 was described for nuclear M2-PK (Lee et al., 2008). The present study underlines that M2-  
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54 PK exists as nuclear protein and suggests a link between the SUMO system and M2-PK.  
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57 Nuclear localization and/or functions have been previously shown for other glycolytic  
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59 enzymes (hexokinase, aldolase, glyceraldehyde 3-P dehydrogenase (GAPDH),  
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phosphoglycerate kinase and enolase) (reviewed in Sirover, 2005). In yeast, hexokinase 2

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3 acts as a nuclear factor in glucose induced transcriptional repression (Rodriguez et al.,  
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5 2001). A nuclear alternative splicing form of enolase 1 has been demonstrated to be involved  
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7 in transcriptional repression of the c-Myc gene in HeLa cells (Ghosh et al., 1999). It was  
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9 shown that GAPDH can modulate the activity of DNA-polymerase- $\alpha$ -primase complex  
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11 (Grosse et al., 1986), and nuclear GAPDH plays a role in control of apoptotic cell death in  
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13 several human cell types (Sawa et al., 1997).  
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For Peer Review

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## Legends to the figures

**Fig. 1. Identification of PIAS3 as M2-PK-binding protein. (A) (Left panel)** Expression of the baits LexA, LexA-M1-PK and LexA-M2-PK in the yeast strain EGY48 was detected by immunoblot analysis using a monoclonal anti-LexA antibody. A lysate of untransformed yeast strain EGY48 was loaded as control. **(Right panel)** Expression of the B42-HA1-PIAS3<sub>383-619</sub> prey fusion protein in EGY48 as shown by immunoblot using anti-HA1 antibodies. **(B)** Derivatives of yeast strain EGY48/pSH1834::URA3 (Mat a, his3, ura3, trp1, leu2::lexA<sub>o6</sub>-pLEU2/lexA<sub>o8</sub>-Gal1-lacZ::URA3) expressing various LexA fusion proteins from pEG202::HIS3 vectors as indicated, were transformed with the plasmid pB42-PIAS3<sub>383-619</sub>::TRP1. Transformants were selected for uracil, histidine, and tryptophane prototrophy and then streaked out onto each of 4 plates and incubated for 4 days at 30°C under the following nutrient conditions: (MM Glu +Leu) Glucose minimal medium with leucine; all strains grow. (MM Glu) Glucose minimal medium without leucine; selection for B42 fusion protein independent activation of the LexA<sub>o6</sub>-LEU2 reporter. (MM Gal/Raf) Galactose minimal medium without leucine, selecting for B42 fusion protein dependent activation of the LexA<sub>o6</sub>-LEU2 gene. (MM Gal/Raf +Leu +X-Gal) Galactose minimal medium with leucine and X-Gal, selecting for B42 fusion protein dependent activation of the LexA<sub>o8</sub>-Gal1-lacZ::URA3. Gal4 served as a positive control and LexA-APP as a negative control.

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3 **Fig. 2. M2-PK interacts with PIAS3 *in vitro*. (A) (left panel)** Purified GST or GST-M2-PK  
4 proteins immobilized on glutathione-Sepharose 4B beads were incubated with whole-cell  
5 extracts from human U-2 OS cells overexpressing the PIAS3 full length protein. The amount  
6 of PIAS3 protein that was retained after washing was determined by immunoblotting using  
7 polyclonal anti-PIAS3 antibodies (top). 5% of the lysate was loaded as input. Input of the  
8 GST proteins was controlled by Coomassie staining (bottom). **(Middle panel)** Purified GST  
9 or GST-PIAS3 proteins immobilized on glutathione-Sepharose 4B beads were incubated with  
10 whole-cell extracts from U-2 OS cells. After washing, the amount of M2-PK protein that was  
11 retained was determined by immunoblotting using a monoclonal anti-M2-PK antibody (top).  
12 5% of the lysate was loaded as input. Input of the GST proteins was controlled by  
13 Coomassie staining (bottom) **(B) Mapping of the PIAS3/M2-PK binding domains. (Left**  
14 **panel)** Purified GST or GST-PIAS3 protein immobilized on glutathione-Sepharose 4B beads  
15 was incubated with extracts from yeast cells expressing B42-HA-M2-PK<sub>1-531</sub> or B42-HA-M2-  
16 PK<sub>1-348</sub> fusion proteins. After washing, the amount of M2-PK protein that was retained was  
17 determined by immunoblotting using a monoclonal anti-M2-PK antibody. M2-PK-input was  
18 loaded as indicated (For input of GST proteins see Fig. 2A). **(Right panel)** Purified GST or  
19 GST-M2-PK protein immobilized on glutathione-Sepharose 4B beads was incubated with  
20 extracts from yeast cells expressing B42-HA-PIAS3<sub>383-619</sub>. After washing, the amount of the  
21 bound B42-HA-PIAS3<sub>383-619</sub> protein was determined by immunoblotting using a monoclonal  
22 anti-HA1 antibody. 5% of the input was loaded (for input of GST proteins see Fig. 2A).  
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3 **Fig. 3. M2-PK interacts with PIAS3 in U-2 OS cell extracts.** Extracts were prepared from  
4 U-2 OS cells transiently overexpressing PIAS3 and subjected to immunoprecipitation with  
5 antibodies against M2-PK, PIAS3 antibodies and control antibodies, as indicated.  
6  
7 Precipitated proteins were separated by SDS-gel electrophoresis and detected by Western  
8 blotting. Total cell lysate (100 µg) was loaded as input control. PIAS3 was  
9 immunoprecipitated by anti-PIAS3 antibodies (left, upper panel) and M2-PK was co-  
10 immunoprecipitated (left, lower panel). M2-PK was immunoprecipitated by M2-PK specific  
11 antibodies (right, lower panel) and PIAS3 was co-immunoprecipitated (right, upper panel).  
12 The species-specific preimmune sera (IP control serum) precipitated neither M2-PK nor  
13 PIAS3.  
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3 **Fig. 4. Sumoylation of M2-PK. (A)** Detection of SUMO-1-M2-PK conjugates in U-2 OS cells.  
4  
5 U-2 OS cells were transiently transfected either with empty vector or with a CMV-promoter  
6 driven expression vector for PIAS3. 30 hours post-transfection, immunoprecipitations were  
7 performed as described in the sumoylation assay with M2-PK specific antibodies from PIAS3  
8 non-overexpressing (lanes 2) or PIAS3 overexpressing U-2 OS cells (lanes 3).  
9 Immunoprecipitations were also performed with an unrelated control serum as negative  
10 control (lane 1). The precipitates were separated by SDS-PAGE and Western blotted using  
11 anti-M2-PK (left panel) and anti-SUMO-1 antibodies (right panel). Input controls are shown  
12 as indicated. (lanes 4). **(B)** Decrease of higher molecular weight M2-PK conjugates in U-2  
13 OS cell lysates over time. Cells were lysed in an SDS containing buffer and the lysates  
14 placed for 6 and 18 hours at 4°C or immediately (0h) processed for Western blotting using  
15 anti-M2-PK antibodies. **(C)** The levels of endogenous and ectopically overexpressed PIAS3  
16 protein were analysed in U-2OS cells by Western blotting using anti-PIAS3 antibodies. **(D)**  
17 M2-PK-SUMO-1 conjugates were co-immunoprecipitated as in figure 4A, and the intensity of  
18 the immunoprecipitated 85 kD band from PIAS3 non-overexpressing and PIAS3  
19 overexpressing U-2 OS cells was quantified by densitometry (Statistical significance was  
20 calculated using Student's t-test, n=4, P= 0.0056 = \*\*).  
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3 **Fig. 5. Subcellular localization of PIAS3 and M2-PK.** U-2 OS or NIH3T3 cells were  
4 transiently transfected with a CMV-promoter driven expression vector for PIAS3 or the empty  
5 vector as indicated. 30 hours later, cells were fixed/permeabilized with 4%PFA/0.2%Triton-X-  
6 100 and stained with anti-PIAS3 antibodies, the DNA-stain TO-PRO-3 or anti-M2-PK  
7 antibodies as indicated and processed for indirect immunofluorescence microscopy and  
8 viewed by using a confocal scanning system. **(A)** Staining of PIAS3-overexpressing U-2 OS  
9 cells with TO-PRO-3 (left panel, red) and anti-PIAS3 antibodies (middle panel, green), Merge  
10 (right panel, yellow). **(B)** Staining of PIAS3-overexpressing U-2 OS cells with TO-PRO-3 (left  
11 panel, red) and anti-M2PK antibodies (middle panel, green), Merge (right panel, yellow). **(C)**  
12 Staining of U-2 OS / empty vector cells with TO-PRO-3 (left panel, red) and anti-M2PK  
13 antibodies (middle panel, green), Merge (right panel, yellow). **(D)** Staining of PIAS3-  
14 overexpressing U-2 OS cells with anti-M2-PK antibodies (left panel, red) and anti-PIAS3  
15 antibodies (middle panel, green), Merge (right panel, yellow). **(E)** Staining of U-2 OS / empty  
16 vector cells with anti-M2-PK antibodies (left panel, red) and anti-PIAS3 antibodies (middle  
17 panel, green), Merge (right panel, yellow). **(F)** Incubation of PIAS3-overexpressing U-2 OS  
18 cells only with the secondary anti-rabbit and anti-goat IgGs **(G)** Staining of PIAS3-  
19 overexpressing NIH3T3 cells with anti-M2-PK antibodies (left panel, red) and anti-PIAS3  
20 antibodies (middle panel, green), Merge (right panel, yellow).  
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3 **Fig. 6. Subcellular localization of endogenous M2-PK in cells overexpressing PIAS3**  
4 **wild type or the catalytically inactive RING domain mutant PIAS3 (C299S, H301A).** U-2  
5  
6 OS cells were transiently transfected with CMV-promoter driven expression vectors for Flag-  
7 mPIAS3 wild type, Flag-mPIAS3 (C299S, H301A) or the empty vector as indicated. 24 hours  
8  
9 later, cells were fixed/permeabilized with 4%PFA/0.2%Triton-X-100 and stained with anti-M2-  
10 PK antibodies and the DNA-stain TO-PRO-3 as indicated and processed for indirect  
11 immunofluorescence microscopy and viewed by using a confocal scanning system. **(A)**  
12 Expression of the Flag-mPIAS3 wild type, Flag-mPIAS3 (C299S, H301A) proteins in U2-OS  
13 cells as shown by immunoblot using anti-Flag antibodies,  $\beta$ -actin served as input control. **(B)**  
14 Staining of Flag-mPIAS3-overexpressing U-2 OS cells with anti-M2-PK antibodies (left panel,  
15 green), TO-PRO-3 (middle panel, red), Merge (right panel, yellow). Cells with nuclear M2-PK  
16 are marked by arrows. **(C)** Staining of mFlag-PIAS3 (C299S, H301A) mutant-overexpressing  
17 U-2 OS cells with anti-M2-PK antibodies (left panel, green), TO-PRO-3 (middle panel, red),  
18 Merge (right panel, yellow). **(D)** Staining of empty vector containing U-2 OS cells with anti-  
19 M2-PK antibodies (left panel, green), TO-PRO-3 (middle panel, red), Merge (right panel,  
20 yellow).

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3 **Supplementary Fig. 1. Predicted sumoylation sites in M2-PK.** (Upper panel) The  
4 predicted sumoylation sites are indicated in the human M2-PK amino acid sequence. (Lower  
5 panel) M2-PK contains eight hypothetical sumoylation motifs. Most SUMO-modified proteins  
6 contain the motif YKXD/E, Y = bulky amino acid, K = lysine residue conjugated to SUMO, X=  
7 any amino acid, D or E = acidic residue. The sumoylation sites in M2-PK were predicted  
8 using [www.abgent.com/doc/sumoplot](http://www.abgent.com/doc/sumoplot).  
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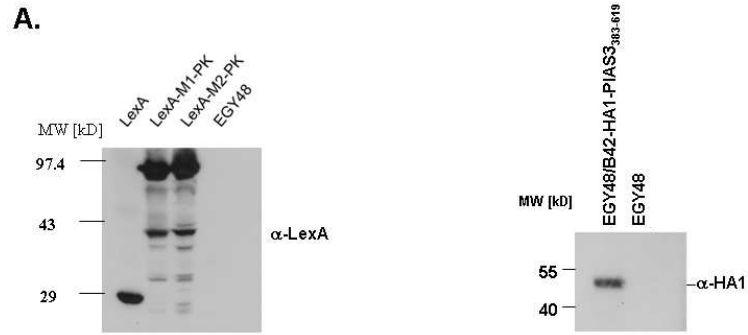


Fig. 1, Spoden et al., 2009

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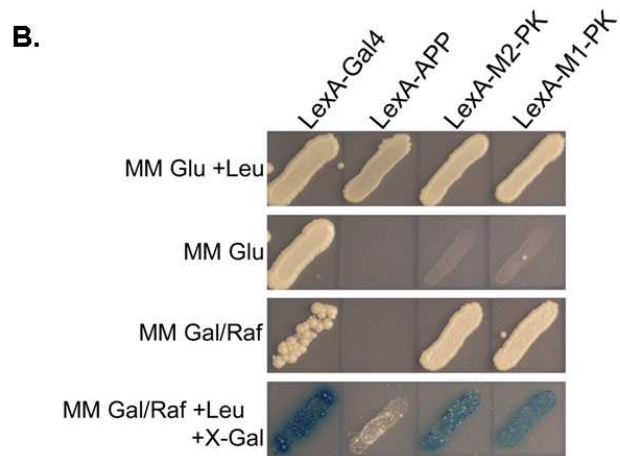


Fig. 1, Spoden et al., 2009, continuation

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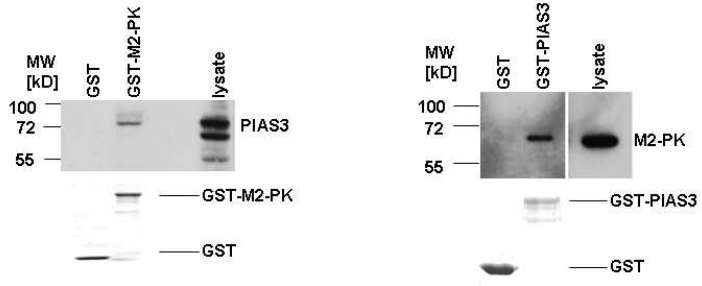


Fig. 2, Spoden et al., 2009

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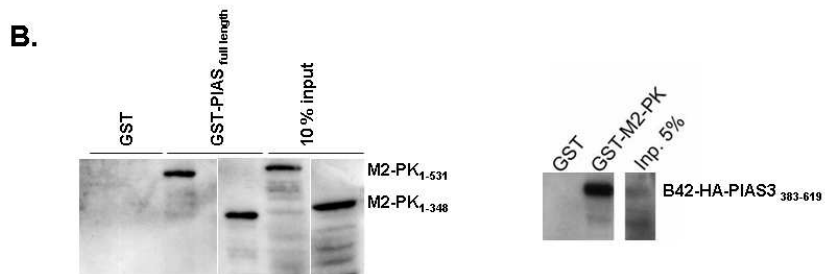


Fig. 2, Spoden et al., 2009, continuation

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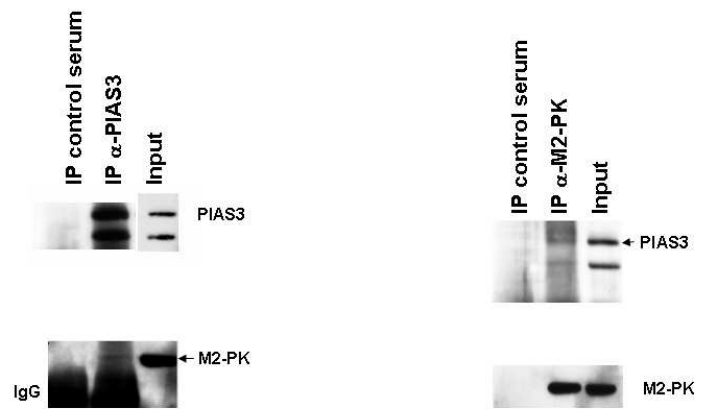


Fig. 3, Spoden et al., 2009

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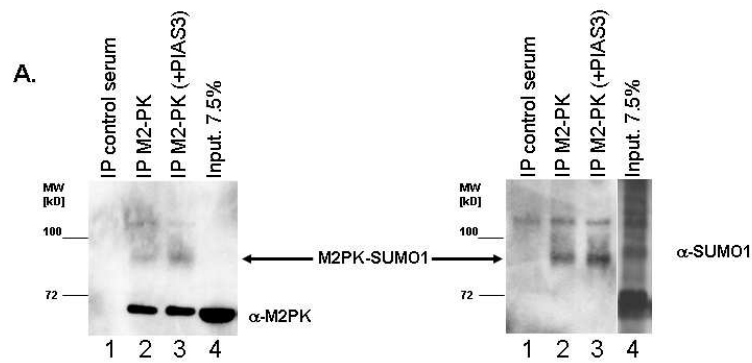


Fig. 4. Spoden et al., 2009

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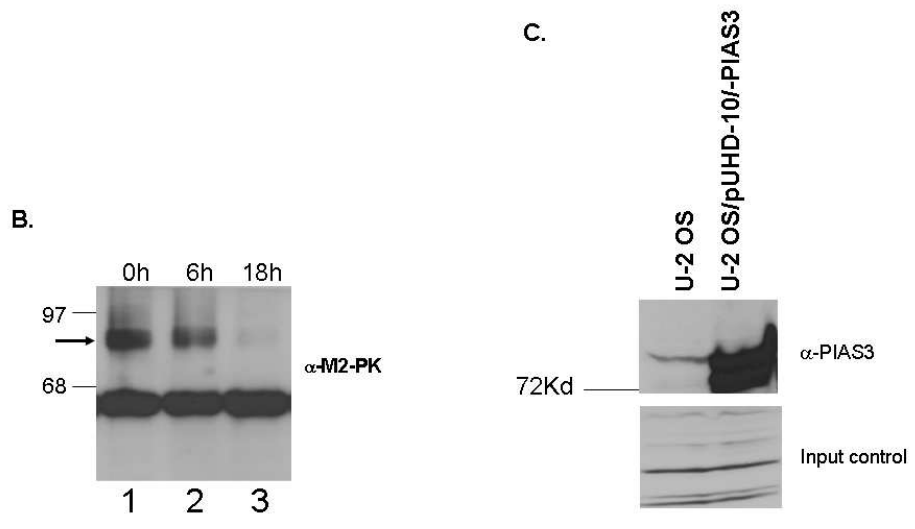


Fig. 4. Spoden et al., 2009, continuation

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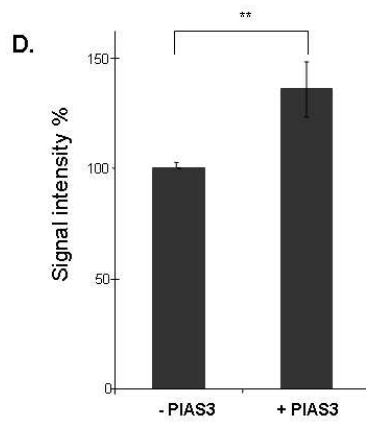


Fig. 4. Spoden et al., 2009, continuation

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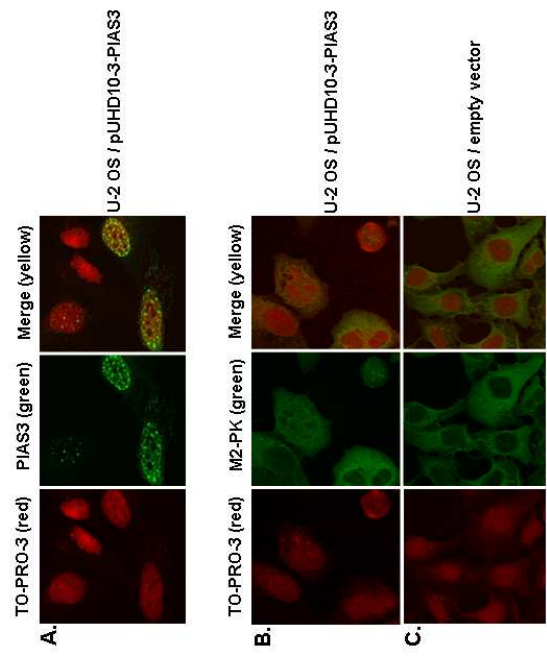


Fig. 5, Spoden et al., 2009

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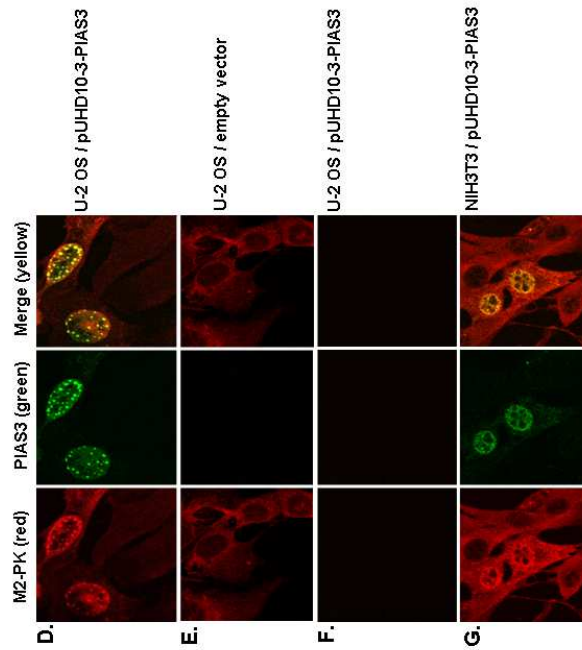


Fig. 5, Spoden et al., 2009, continuation

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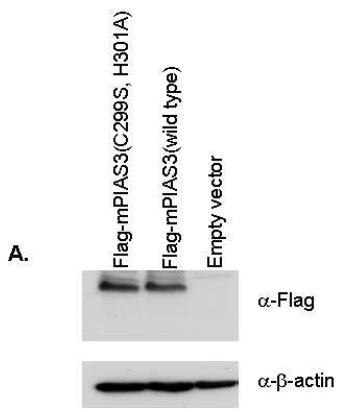


Fig. 6, Spoden et al., 2009

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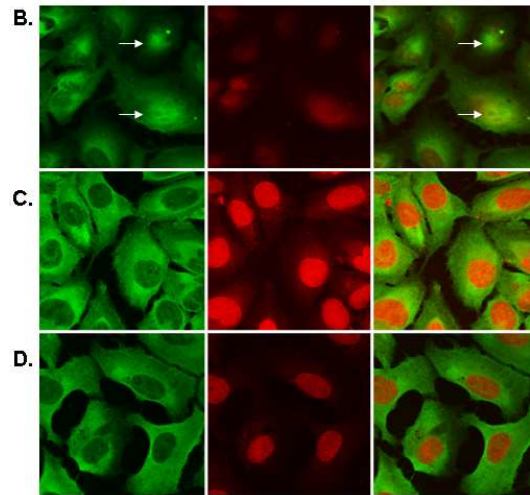


Fig. 6, Spoden et al., 2009, continuation

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201 GSLGSKRGVN LPGAAVDLPA VSEKDIQDLK FGVEQVDVMV FASFIRKASD

251 VHEVRKVLGE KGMNIKIISK IENHEGVRRF DEILEASDGI MVARGDLGIE

301 IPAEKVFLAQ KMMIGRCNRA GKFVICATQM LESMIKKFRP TRAEGSDVAN

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501 GFFKKGDVVI VLTGWRFSGG FTNIMRVVEV P

■ Motifs with high probability

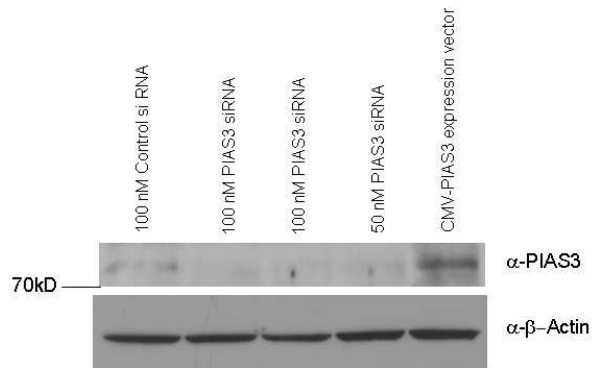
■ Motifs with low probability

■ Overlapping Motifs

No.	Pos.	Group	Score	No.	Pos.	Group	Score
1	K336	MLESM <b>IKKP</b> RPTRA	0.84	6	K504	KARGF <b>FKKG</b> DVVIV	0.68
2	K367	LSGET <b>AKGD</b> YPLEA	0.79	7	K151	DNAYM <b>EKCD</b> ENILW	0.50
3	K66	TLKEM <b>IKSG</b> MNVAR	0.77	8	K505	ARGFF <b>KKGD</b> VVIVL	0.48
4	K230	KDIQD <b>LKFG</b> VEQDV	0.73				
5	K135	TAEVE <b>LKKG</b> ATLKI	0.73				

Supplementary Fig. 1, Spoden et al., 2009

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Accompanying Fig.1: U-2 OS cells were transiently transfected with siRNAs or a CMV-PIAS3 expression vector, as indicated. In PIAS3 siRNA experiments, 50 nM or 100 nM for human PIAS3 (ON-TARGETplus SMARTpool L-004164-00-0005, Human PIAS3, NM\_006099; Dharmacon) or siControl RNA (D-001210-01; Dharmacon) was transfected. The cell lysates were harvested 48 hours after transfection and subjected to Western blotting to detect PIAS3 and  $\beta$ -actin expression levels using anti-PIAS3 (AP1245; Abgent) and anti- $\beta$ -actin (Sigma) antibodies.

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## The SUMO-E3 ligase PIAS3 targets Pyruvate kinase M2

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Martin Hermann<sup>4</sup>, and Werner Zwerschke<sup>1,2,\*</sup>

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<sup>4</sup>KMT Laboratory, Department of General and Transplant Surgery, Innsbruck Medical University, A-6020 Innsbruck, Austria.

Running head: M2-PK interacts with PIAS3.

Key words: Glycolysis, pyruvate kinase, PKM2, SUMO E3-ligase, PIAS3, sumoylation.

Total number of text figures & tables: 5

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**Abstract**

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Pyruvate kinase M2 (M2-PK) controls the rate-limiting step at the end of the glycolytic pathway in normal proliferating and tumor cells. Other functions of M2-PK in addition to its role in glycolysis are little understood. The aim of this study was to identify new cellular interaction partners of M2-PK in order to discover novel links between M2-PK and cellular functions. Here we show that the SUMO E3 ligase protein PIAS3 (inhibitor of activated STAT3) physically interacts with M2-PK and its isoenzyme M1-PK. Moreover, we demonstrate that endogenous SUMO-1-M2-PK conjugates exist in mammalian cells. Furthermore, we show that transient expression of PIAS3 but not the RING domain mutant PIAS3 (C299S, H301A) is consistent with nuclear localization of M2-PK and PIAS3 and M2-PK partially colocalize in the nucleus of these cells. This study suggests a link between PIAS3 and nuclear pyruvate kinase.

## Introduction

Pyruvate kinase (PK, ATP:pyruvate O'-phosphotransferase, EC 2.7.1.40) is one of the rate-controlling glycolytic enzymes that catalyze the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, to generate pyruvate and ATP (Staal and Rijksen, 1991). Four distinct pyruvate kinase isoenzymes (L, R, M1, and M2) occur in mammalian cells. M2-PK (alias PKM2) is the embryonic form that replaces the other isoforms in normal proliferating and in tumour cells (Mazurek et al., 2005). The human M1 and M2 isoenzymes are encoded by one gene; the specific mRNAs are generated by alternative splicing of the M1 specific exon 9 and the M2 specific exon 10, respectively (Takenaka et al., 1991). M2-PK is subject to allosteric activation by fructose-1,6-bisphosphate (FBP), an intermediate metabolite in the glycolytic pathway. Activation of M2-PK involves a shift from a dimeric to a tetrameric conformation. Tetrameric M2-PK is characterized by a high affinity to its substrate PEP and high activity at physiological PEP concentrations, whereas dimeric M2-PK has a low PEP affinity and is nearly inactive under physiological conditions (Mazurek et al., 2005). The dimer-tetramer switch of M2-PK contributes to the regulation of the glycolytic flux in proliferating cells and has been shown to be regulated by direct interaction with viral and cellular oncoproteins (Zwerschke et al., 1999; Mazurek et al., 2001), suggesting that M2-PK activity is tightly controlled in tumour cells. M2-PK is predominantly detected in many if not all human cancers (Mazurek et al., 2005) and lung cancer cells predominantly expressing M2-PK grow to large tumours in a nude mice xenografts model (Christofk et al., 2008). These data suggest that M2-PK plays an important role for tumor growth. The tetrameric form of M2-PK is associated with other glycolytic enzymes as well as with lactate dehydrogenase, nucleotide diphosphate kinase, and adenylate kinase, within a protein complex, referred to as the glycolytic enzyme complex (Mazurek et al., 1996; Mazurek et al., 2001). In contrast, dimeric and also a monomeric form of M2-PK are not associated with the glycolytic complex (Mazurek et al., 1996). Monomeric M2-PK was described as a cytosolic thyroid hormone binding protein (Kato et al., 1989), and, recently nuclear localization and a role in

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3 transcriptional regulation was described for M2-PK (Lee et al., 2008); however, non-glycolytic  
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5 functions of M2-PK are little understood.  
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10 SUMO, a small ubiquitin-like modifier protein that is highly conserved from yeast to human, is  
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12 conjugated to a large number of cellular target proteins (Uzunova et al., 2007). Similar to  
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14 enzymatic ubiquitination, the conjugation of specific SUMO proteins (SUMO-1-3) to target  
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16 proteins requires an E1-activating enzyme (Aos1/Uba2) as well as an E2-type SUMO-1-  
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18 conjugating enzyme (Ubc9; Johnson, 2004). More recently, proteins that increase the  
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20 efficiency of SUMO conjugation have been discovered. The members of the mammalian  
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22 PIAS (Protein Inhibitor of Activated STAT) protein family, originally identified as cytokine-  
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24 induced inhibitors of the STAT family of transcription factors (Chung et al., 1997), belong to  
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26 this protein class, referred to as SUMO E3 ligases. The PIAS family consists of seven  
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28 structurally related proteins (PIAS1, PIAS3, PIAS3 $\beta$ , PIASx $\alpha$ , PIASx $\beta$ , PIASy and PIASyE6)  
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30 (Shuai and Liu, 2005). Similar to many ubiquitin E3 ligases, these proteins contain a  
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32 predicted RING-finger-like structure which has been shown to be essential for their SUMO  
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34 E3 ligase activities toward various target proteins. In contrast to the well-known function of  
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36 polyubiquitin conjugation in proteasome-mediated protein degradation, sumoylation seems to  
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38 be rarely involved in protein degradation processes. There is evidence that SUMO-2/3  
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40 conjugates in human cells are controlled via degradation by the proteasome while SUMO-1  
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42 conjugates show little changes upon inhibition of the proteasome (Uzunova et al., 2007).  
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44 Sumoylation is a highly dynamic process and its outcomes are highly diverse, ranging from  
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46 changes in subcellular localization, transcriptional regulation to altered activity and stability of  
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48 the modified protein (Johnson, 2004). PIAS proteins do not operate merely as SUMO E3s,  
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50 since their co-regulator effects are often independent of their RING finger but dependent on  
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52 their SIM (SUMO-interacting motif) or SAP (scaffold attachment factor-A/B/acinus/PIAS)  
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54 domain (Palvimo, 2007).The modulator activity of the PIAS/SUMO system has so far mainly  
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56 been shown to involve altered subnuclear targeting and/or assembly of transcription  
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5 The aim of this study was to discover novel cellular M2-PK-binding proteins. We identified  
6 the SUMO E3 ligase PIAS3 (Chung et al., 1997) as a novel M2-PK-interacting protein and  
7 analyzed functional consequences of this interaction.  
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## Materials and Methods

### *Interaction analysis in yeast*

LexA-M2-PK was used as bait in the yeast two-hybrid screen to identify cDNAs for M2-PK-binding proteins from a human WI38 fibroblast cDNA expression library (pB42-WI38 cDNA::TRP1) (Zwerschke et al., 1999). The pB42-WI38 cDNA::TRP1 plasmids express the B42 transactivation domain-prey fusions proteins from the inducible GAL1 promoter. Yeast strain EGY48/pSH1834 (MATa, his3, ura3, trp1, leu2::lexA<sub>o6</sub>-pLEU2/LexA<sub>o8</sub>-GAL1-lacZ::URA3) was used for both the LEU2 and  $\beta$ -galactosidase reporter gene assays. For determination of reporter gene activity, EGY48/pSH1834 was transformed with pEG202::HIS3 plasmids expressing the LexA-M2-PK, LexA-M1-PK or LexA-APP<sub>599-695</sub> fusion protein, together with the plasmid pB42-HA-PIAS3<sub>383-619</sub>::TRP1, and it was selected for leucine prototrophy as previously described (Zwerschke et al., 1999). Alternatively,  $\beta$ -galactosidase activity was determined in cellular extracts as previously described (Zwerschke et al., 1996). The various LexA fusion proteins were expressed to the same level, as confirmed by direct immunoblotting, using a polyclonal antibody against LexA.

### *Plasmids*

The complete human M2-PK cDNA was isolated by EcoRI/XhoI restriction digest from the yeast prey vector pB42-M2-PK::TRP1 (Zwerschke et al., 1999) and inserted in frame to GST in the bacterial expression vector pGEX-4T-1 (Amersham Biosciences, Vienna, Austria) to generate pGEX-4T-1/M2-PK, and fused in frame to LexA in the yeast two-hybrid bait vector pEG202 (Zwerschke et al., 1999) to generate the bait vector pEG202/M2-PK. To construct the bait vector pEG202/APP<sub>599-695</sub>, the human APP cDNA encoding for the amino acids 599-695 was amplified by PCR and inserted as an EcoRI/NotI fragment in frame to LexA into pEG202. To generate pGEX-4T-1/M1-PK and pEG202/M1-PK the sequence encoding the M2-PK specific region was exchanged with the M1-PK specific region isolated as SphI/XhoI fragment from the vector pX/M1-PK (Mazurek et al., 1996). The yeast expression vector

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3 pEG202/M2-PK<sub>1-348</sub> encoding for the carboxyl-terminal deletion mutant of M2-PK from amino  
4 acid residues 1-348 fused to the B42 transactivation domain was constructed by the deletion  
5 of a MscI/XhoI fragment in pEG202/M2-PK spanning from the 5' MscI side to the stop codon  
6 of M2-PK. The human PIAS3 cDNA was amplified from the RZPD clone  
7 IDIMAGp958J08198Q2 (Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin,  
8 Germany) by PCR and ligated as EcoRI/EcoRI fragment into the EcoRI site of the pEG202,  
9 pGEX-4T-1 and pUHD10-3 to generate pEG202/PIAS3, pGEX-4T-1/PIAS3 and pUHD10-  
10 3/PIAS3 respectively. All plasmids were confirmed by sequencing. The plasmids Flag-  
11 mPIAS3 wild type and Flag-mPIAS3 (C299S, H301A) are gifts of Fang Liu (see, Long et al.,  
12 2004).

#### 23 24 25 26 27 *In vitro interaction analysis*

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29 GST pull down assays were performed essentially as described (Mannhardt et al., 2000),  
30 using 5 µg purified GST, GST-M2-PK or GST-PIAS3 proteins and purified PIAS3 protein or  
31 U-2 OS cell lysates. PIAS3 protein was purified by immunoprecipitation using anti-PIAS3  
32 antibodies (AP,1245a, Abgent, Heidelberg, Germany) and protein A and G agarose beads.  
33 The beads were washed and the purified PIAS3 protein was eluted using 0.1 M glycine HCl,  
34 pH 3.5, into vials containing 1 M Tris, pH 8.0. The purified PIAS3 protein (<200ng) was either  
35 incubated with purified GST-M2-PK (800 ng) or GST (1000 ng).  
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#### 46 47 *Coimmunoprecipitation experiments*

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49 Co-immunoprecipitation experiments were performed essentially as described (Mannhardt et  
50 al., 2000).  
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#### 55 56 *Western blotting experiments*

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58 Immunoblot analysis was performed essentially as described (Mannhardt et al., 2000) using  
59 antibodies against LexA (sc-7544, IgG1, mouse monoclonal Santa Cruz Biotechnology,  
60 Vienna, Austria), M2-PK (clone DF4, mouse monoclonal, ScheBo Biotech, Wetttenberg,

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3 Germany), SUMO-1 (33-2400, mouse monoclonal, Zymed, Vienna, Austria, or A-712, rabbit  
4 polyclonal, BostonBiochem, USA), M1/2-PK (200-1178, goat polyclonal, Rockland, Vienna,  
5 Austria), HA1 (sc-805, rabbit polyclonal, Santa Cruz Biotechnology) and PIAS3 (AP,1245a,  
6 rabbit polyclonal, Abgent). Further antibodies used: anti-rabbit, W401B, IgG, HRP conjugate;  
7 anti-mouse W4021, IgG, HRP conjugate; anti-goat, W4011, IgG, HRP conjugate (Promega,  
8 Vienna, Austria). Anti-goat IgG (FITC-conjugate Jackson, Vienna, Austria). Anti-rabbit IgG,  
9 TRITC-conjugate (DAKO, Vienna, Austria).  
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### 20 *In vivo sumoylation assay*

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22 To prevent desumoylation of the target proteins, U-2 OS cells were resuspended in SUMO  
23 immunoprecipitation buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2% SDS) and incubated  
24 at 95°C for 10 minutes to inactivate desumoylating enzymes (Buschmann et al., 2001). The  
25 lysate was diluted 1:10 in dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton-  
26 X-100) and centrifuged at 20,000g and 4°C. After preclearing with A/G agarose beads (50  
27 µg/1 mg lysate) M2-PK was precipitated over night with goat polyclonal antibodies against  
28 pyruvate kinase (Rockland Immunochemicals, Gilbertsville, PA) and A/G agarose beads. The  
29 precipitates were washed with dilution buffer, separated by SDS-PAGE and blotted onto a  
30 PVDF membrane. The PVDF membrane was probed with monoclonal anti-M2-PK or  
31 monoclonal anti-SUMO-1 antibodies.  
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### 48 *Cell culture and transfection*

49 The human osteosarcoma cell line U-2 OS was cultured in DMEM plus 10% fetal calf serum  
50 as described (Mannhardt et al., 2000) and the mouse NIH3T3 cell line was cultured in DMEM  
51 plus 10% calf serum as described (Zwerschke et al., 1999). Transient transfection of the  
52 expression vector pUHD-3/PIAS3 was performed using Effectene (Qiagen, Hilden,  
53 Germany).  
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### *Indirect immunofluorescence analysis*

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3 Indirect immunofluorescence analysis was performed essentially as described previously  
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5 (Mannhardt et al., 2000). Briefly, cells were grown to about 80% confluence on glass cover  
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7 slips and transiently transfected (Effectene, Qiagen, Germany). At 30 h post transfection,  
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9 cells were prepared for indirect immunofluorescence according to standard protocols,  
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11 including fixation with 4%PFA/0.2% Triton-X-100 and incubation with primary antibodies ( $\alpha$ -  
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13 PIAS3 antibody, Abgent, clone AP1245a, Vienna;  $\alpha$ -M2-PK antibody, Rockland, Vienna) and  
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15 TRITC- or FITC-conjugated secondary antibodies. Nuclear staining was conducted with TO-  
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17 PRO-3 (Molecular Probes, Göttingen, Germany). Samples were viewed by indirect  
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19 immunofluorescence microscopy using the confocal scanning system MicroRadiance (Bio-  
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21 Rad, Hempstead, U.K.) in combination with a Zeiss Axiophot microscope. The following  
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23 filters were used for FITC-derived and TRITC-derived fluorescence: excitation for FITC 488  
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25 nm, TRITC 543 nm and emission for FITC 515 to 530 nm and TRITC >570 nm.  
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## Results

### Identification of PIAS3 as M2-PK binding protein

To better understand the function of M2-PK, a yeast two-hybrid screen for cellular M2-PK-binding proteins was conducted. We used full length M2-PK fused in frame to the LexA-DNA-binding domain as bait (Zwerschke et al., 1999). LexA-M2-PK can bind to the LexA binding sites of a synthetic LEU2 reporter gene, and thereby allows monitoring of the interaction between M2-PK and a second hybrid protein containing the B42 transactivation domain fused in-frame to a heterologous cDNA. When a galactose inducible human fibroblast cDNA expression library (pB42-WI38 cDNA::TRP1) was coexpressed with LexA-M2-PK in the yeast strain EGY48/pSH1834 and screened for transformants that grew on leucine-deficient medium, cDNAs encoding M2-PK and fragments thereof were repeatedly isolated (G. Spoden and W. Zwerschke, unpublished findings), reflecting the tendency for M2-PK to form tetramers and dimers (Zwerschke et al., 1999). Intriguingly, the M2-PK bait identified a cDNA encoding for the intact carboxyl-terminal substrate binding domain (amino acids 383-619) of protein inhibitor of activated STAT3 (PIAS3) (Chung et al., 1997), which was more recently identified as a SUMO E3 ligase involved in SUMO-1 modification and transcriptional repression of interferon regulatory factor-1 (Nakagawa and Yokosawa, 2002) and microphthalmia transcription factor (Miller et al., 2005). Coexpression of the PIAS3<sub>383-619</sub>-HA1-B42 fusion protein with LexA-M2-PK (Fig. 1A) enabled the yeast strain EGY48/pSH1834 to grow on galactose but not glucose minimal plates, indicating that leucine prototrophy depends on expression of the M2-PK-B42 fusion protein (Fig. 1B). PIAS3 also bound to the M1-PK isoenzyme in this assay (Fig. 1B), suggesting that the M2-PK specific region is not necessary for the pyruvate kinase/PIAS3 interaction. The inability of the unrelated fusion protein LexA-APP<sub>599-695</sub> to bind to B42-HA-PIAS3<sub>383-619</sub> indicates that the M-type pyruvate kinase sequences are essential for binding to PIAS3. These findings were corroborated using lacZ as a second reporter gene. High  $\beta$ -galactosidase activity (blue color)

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3 was observed when B42-HA-PIAS3<sub>383-619</sub> was coexpressed with either LexA-M2-PK or LexA-  
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5 M1-PK, but not with the unrelated fusion protein LexA-APP<sub>599-695</sub> (Fig. 1B, bottom panel).  
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10 To analyze whether M2-PK also interacts with the PIAS3 full-length protein, purified GST-  
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12 M2-PK was incubated with extracts from human U-2 OS cells overexpressing wild type  
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14 PIAS3. PIAS3 specifically bound to GST-M2-PK in this assay, whereas no binding was  
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16 observed for GST (Fig. 2A, left). Moreover, the incubation of GST-PIAS3 full-length protein  
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18 with lysates from human U-2 OS cells demonstrates that human M2-PK binds specifically to  
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20 purified GST-PIAS3, but not to GST alone (Fig. 2A, right). These data suggest that M2-PK  
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22 interacts with PIAS3 in vitro. To map the PIAS3-interacting region on M2-PK, we incubated  
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24 GST-PIAS3 either with the M2-PK full-length protein (aa 1-531) or the carboxyl-terminal  
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26 deletion mutant M2-PK<sub>1-348</sub> lacking the M2-PK specific region (Fig. 2B, left). Both proteins  
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28 were expressed in yeast cells and the lysates incubated with purified GST-PIAS3 and GST.  
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30 We found that M2-PK<sub>1-348</sub> bound as strongly as the M2-PK full-length protein to GST-PIAS3  
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32 (Fig. 2B, left). This underlines the specificity of the PIAS3/M2-PK protein-protein interaction  
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34 and suggests that M2-PK<sub>1-348</sub> is sufficient for PIAS3 binding. This is in keeping with the  
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36 finding that the B42-HA-PIAS3<sub>383-619</sub> protein specifically interacts with both M-type pyruvate  
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38 kinase isoenzymes (Fig. 1), whose amino acid sequences are identical in this region. The  
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40 interaction of M2-PK with the intact carboxyl-terminal substrate binding domain of the PIAS3  
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42 protein found in living yeast cells (Fig. 1) was corroborated in a GST pull down experiment.  
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44 GST-M2-PK bound specifically and with high affinity to B42-HA-PIAS3<sub>383-619</sub> (Fig. 2B, right),  
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46 underlining that it is the C-terminal domain of PIAS3 that interacts with M2-PK. Collectively,  
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48 these results map the interaction domains to the amino-terminal part of M2-PK (residues 1-  
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50 348) and the carboxyl-terminal domain of PIAS3 (residues 383-619).  
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57 To show that the described interaction also occurs in cell extracts, co-immunoprecipitation  
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59 experiments were conducted using human U-2 OS cells transiently transfected with a CMV  
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promoter driven PIAS3 expression vector (Fig. 3). Endogenous M2-PK was specifically co-

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3 precipitated with PIAS3 using anti-PIAS3 antibodies, but not with an unrelated polyclonal  
4 control serum (Fig. 3, left). M2-PK antibodies precipitated M2-PK from U-2 OS extracts and  
5 co-immunoprecipitation of PIAS3 was observed, whereas an unrelated polyclonal control  
6 serum precipitated neither M2-PK nor PIAS3 (Fig. 3, right). These findings suggest that M2-  
7 PK can form a complex with the SUMO E3 ligase PIAS3 in extracts from human cells.  
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### 16 **A subfraction of M2-PK is SUMO-1-conjugated**

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18 Computer-aided analysis of the human M2-PK sequence revealed eight putative SUMO-1  
19 acceptor sequences in the M2-PK molecule (supplementary Fig. 1), suggesting that it may  
20 be a sumoylation target. To examine whether endogenous M2-PK is covalently conjugated  
21 with SUMO-1, U-2 OS cell extracts were prepared in SDS-containing lysate buffer to avoid  
22 desumoylation, and M2-PK was immunoprecipitated from the extracts overnight. The  
23 precipitates were analyzed by Western blotting with antibodies against M2-PK and SUMO-1,  
24 respectively (Fig. 4A). The anti-M2-PK antibody recognized a specific band at 58 kD,  
25 corresponding to unmodified M2-PK, and two slower migrating bands at approximately 80  
26 and 110 kD (Fig. 4A, left, lane 2). The anti-SUMO-1 antibody recognized the two slower  
27 migrating bands at 80 and 110 kD (Fig. 4A, right, lane 2). The 110 kD protein was also  
28 immunoprecipitated by isotypic control antibodies (Fig. 4A, right, lane 1), indicating that only  
29 the 58 and 85 kD bands are specifically immunoprecipitated by the anti-M2-PK antibodies.  
30 This data suggests that the 80 kD band is consistent with the conjugation of SUMO-1 to  
31 endogenous M2-PK. Most of the protein recognized by the anti-M2-PK antibodies runs at 58  
32 kD (Fig. 4A), suggesting that only a part of the endogenous M2-PK exists as SUMO-1  
33 conjugate and/or that the M2-PK-SUMO-1 conjugates are relatively unstable. To address this  
34 question, the U-2 OS cells were harvested in a stabilizing SDS-containing lysis buffer and  
35 either immediately processed for Western blotting (Fig. 4B, lane 1) or incubated for six (Fig.  
36 4B, lane 2) or eighteen (Fig. 4B, lane 3) hours at 4°C before processing. Over time the  
37 intensity of the high molecular weight bands recognized by the anti-M2-PK antibody was  
38 reduced (Fig. 4B, compare lanes 1, 2 and 3) - especially after eighteen hours, the incubation  
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3 time used for the immunoprecipitation experiment in Fig. 4A. These data suggest that higher  
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5 molecular weight M2-PK conjugates exist, but that these proteins are relatively unstable,  
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7 explaining the weak signals obtained in the Co-IP (Fig. 4A).  
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11 To analyze whether PIAS3 specifically enhances M2-PK sumoylation, PIAS3 was transiently  
12 expressed in U-2 OS cells (Fig. 4C) and M2-PK was immunoprecipitated from PIAS3-  
13 overexpressing U-2 OS cells. When the immunoprecipitates were probed with anti-M2-PK  
14 (Fig. 4A, left, lane 3) and anti-SUMO-1 (Fig. 4A, right, lane 3) antibodies, it was found that  
15 the intensity of the 80 kD M2-PK-SUMO-1 conjugate was slightly increased in the PIAS3 co-  
16 expressing cells. The triple repetition of this immunoprecipitation experiment and  
17 quantification of the intensity of the 80 kD M2-PK-SUMO-1 band from PIAS3 non-  
18 overexpressing and PIAS3 overexpressing U-2 OS cells by densitometry showed that PIAS3  
19 produced a slight increase in M2-PK sumoylation (Fig. 4D).  
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### 33 **Expression of PIAS3 coincides with nuclear localization of M2-PK**

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35 Since there is precedence that sumoylation can influence the subcellular localization of target  
36 proteins acting in carbohydrate metabolism, as has been shown for the glucose transporter  
37 GLUT4 and GLUT1 (Giorgino et al., 2000; Lalioti et al., 2002), we investigated the subcellular  
38 localization of M2-PK in PIAS3 overexpressing cells. To this end, we transiently transfected a  
39 PIAS3 expression vector into U-2 OS cells. Costaining of PIAS3 with anti-PIAS3 antibodies  
40 and the DNA stain TO-PRO-3 revealed that PIAS3 (green) was predominantly localized in  
41 the nucleus (red), a nuclear speckled staining for PIAS3 was found, as monitored by indirect  
42 immunofluorescence experiments using a confocal laser-scanning microscope (Fig. 5A).  
43 Costaining of M2-PK and TO-PRO-3 revealed that M2-PK (green) was also localized in the  
44 nucleus (red) in PIAS3 overexpressing cells (Fig. 5B); however, not in U-2 OS cells  
45 containing only empty vector (Fig. 5C). To analyze whether M2PK and PIAS3 colocalize in  
46 the nucleus of PIAS3 overexpressing U-2 OS cells, we conducted a costaining with anti-M2-  
47 PK and anti-PIAS3 antibodies and visualized the staining by either red (M2-PK) or green  
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3 (PIAS3) fluorescence (Fig. 5D). U-2 OS cells transiently expressing PIAS3 (Fig. 5D, middle,  
4 green) showed a strong nuclear staining signal for M2-PK, although significant levels of M2-  
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6 PK were still detectable in the cytoplasm (Fig. 5D, left). This is consistent with the finding that  
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8 only a part of endogenous M2-PK is SUMO-1 conjugated (Fig. 4). In the PIAS3  
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10 overexpressing cells, M2-PK was distributed throughout the nucleus, whereas PIAS3 was  
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12 predominantly detected in defined nuclear speckles, and colocalization between M2-PK and  
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14 PIAS3 was observed predominantly at these nuclear structures (Fig. 5D, right, yellow). In  
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16 mock-transfected U-2 OS cells, the anti-M2-PK antibodies stained only structures in the  
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18 cytoplasm but not in the nucleus, and endogenous PIAS3 was not detectable (Fig. 5E). No  
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20 staining of U-2 OS cells was observed when only the secondary anti-rabbit and anti-goat  
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22 IgGs were added (Fig. 5F). To corroborate these findings, PIAS3 was transiently expressed  
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24 in NIH3T3 mouse fibroblasts. Similar to our findings in U-2 OS cells, ectopic expression of  
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26 PIAS3 resulted in colocalization of PIAS3 with M2-PK in the nucleus of NIH 3T3 cells (Fig.  
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28 5G). Together these experiments demonstrate that PIAS3 and M2-PK specifically co-localize  
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30 in the nucleus in PIAS3 overexpressing cells.  
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36 To test whether the increased nuclear localization of M2-PK depends on the catalytically  
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38 active RING domain in PIAS3, we employed the catalytically inactive PIAS3 RING domain  
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40 mutant Flag-mPIAS3 (C299S, H301A) described in Long et al., (2004), and investigated the  
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42 impact of the Flag-mPIAS3 RING domain mutant and of Flag-mPIAS3 wild type on nuclear  
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44 localization of M2-PK in U2-OS cells (Fig.6). We transiently expressed both proteins in U2-  
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46 OS cells and detected nuclear M2-PK only in the Flag-mPIAS3 (wild type) (Fig. 6B) but not in  
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48 the Flag-mPIAS3 (C299S, H301A) overexpressing cells (Fig.6C). For this reason, we  
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50 conclude that the intact RING domain in PIAS3 is important for nuclear localization of M2-PK.  
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## Discussion

In this study we have identified the SUMO E3 ligase PIAS3 (Chung et al., 1997) as interaction partner of the glycolytic isoenzymes M2-PK and M1-PK in a two-hybrid screen. The interaction between M2-PK and PIAS3 was further corroborated in GST-pull down assays and by co-immunoprecipitation of PIAS3 and M2-PK from extracts of human U-2 OS cells. Moreover, we found that a substantial fraction of endogenous M2-PK is covalently conjugated to SUMO-1 and we present data that M2-PK colocalizes with PIAS3 in nuclear structures in cells transiently overexpressing PIAS3. These data suggest a functional link between PIAS3 and M2-PK.

### M2-PK as sumoylation substrate

Since SUMO-1 has an apparent molecular mass of approximately 20 kD (Matunis et al., 1996) and the monosumoylation of RanGAP1 increases its apparent MW by roughly 25 kD (Matunis et al., 1996), our identification of an additional M2PK band with an apparent MW of 80 kD in Fig. 4, which is also recognized by SUMO-1 antibodies, is consistent with the addition of one SUMO-1 per M2-PK. Using the SUMO plot program ([www.abgent.com/doc/sumoplot](http://www.abgent.com/doc/sumoplot)), we identified eight potential sumoylation motifs (YKXD/E; Y bulky amino acid, K attachment site lysine, X any amino acid) in M2-PK (supplementary Fig. 1). Moreover, analysis of the X-ray structure of human M2-PK (Dombrauckas et al., 2005) showed that all eight lysine residues are exposed on the surface of the enzyme. This underlines our finding that M2-PK is sumoylated. The identification of the sumoylated lysine residue(s) in M2-PK requires further study.

The identification of human pyruvate kinase as target for SUMO-1 conjugation in this study is reminiscent of similar findings in yeast. Thus, the *S.cerevisiae* homolog of pyruvate kinase Pyk1/Cdc19 has been identified as a SUMO substrate in mass spectrometry-based proteome-wide approaches (Hannich et al. 2004, Zhou et al. 2004, Panse et al. 2004, Wohlschlegel et al., 2004, Denison et al. 2005), suggesting that sumoylation of pyruvate kinase, as described here, represents an evolutionarily conserved process.



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3 The two human M-type pyruvate kinase isoenzymes consist of 531 amino acid residues and  
4 differ only in 22 amino acid residues in a 45 residues-long domain encoded by the respective  
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The two human M-type pyruvate kinase isoenzymes consist of 531 amino acid residues and differ only in 22 amino acid residues in a 45 residues-long domain encoded by the respective exons (Dombrauckas et al., 2005). The type-specific domains are important for the interaction of the M1 and M2 pyruvate kinase subunits, respectively, and although structurally very similar (Wooll et al., 2001; Dombrauckas et al., 2005), the non-allosteric M1 isoenzyme exists only as tetramer, while M2-PK is an allosteric enzyme that can exist in different conformations. All hypothetical sumoylation motifs are localized in domains common to M1- and M2-PK (supplementary Fig. 1), suggesting that M1-PK may also be subject to sumoylation. This is in keeping with our finding that PIAS3 specifically binds to both isoenzymes, M2-PK and M1-PK in a yeast two-hybrid assay (Fig. 1), and further underlined by our finding that the amino-terminal domain, which is common to both M-type isoenzymes, is sufficient for the PIAS3/pyruvate kinase interaction (Fig. 2). While these findings suggest that PIAS3 interacts with both M1-PK and M2-PK subunits, one cannot rule out that the quaternary structure of pyruvate kinase influences the strength of the M2-PK/PIAS3 interaction. It is conceivable that also the other two pyruvate kinase isoenzymes, L-PK and R-PK, can exist as sumoylated proteins in cells expressing these PK isoforms. This question needs, however, further studies.

### **M2-PK as PIAS3 target protein**

We found that overexpression of PIAS3 induces a moderate increase in the sumoylation of M2-PK. This is in line with the finding that only a fraction of the endogenous M2-PK was detected in a complex with PIAS3 in the co-immunoprecipitation experiments and modified by SUMO. These results suggest that PIAS3 enhances sumoylation of M2-PK, but that the M2-PK/PIAS3 interaction occurs between a rather small fraction of the two proteins. However, the SUMO E2 enzyme Ubc9 can directly bind the consensus sequence YKXD/E in a given substrate and Ubc9 together with the SUMO E1 enzyme are sufficient to efficiently sumoylate substrates *in vitro* in the absence of an E3 (Johnson, 2004; Reverter and Lima, 2005), indicating that E3 enzymes can enhance sumoylation without being indispensable.

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3 Moreover, there are examples in which the effect of PIAS proteins on substrate function is  
4 independent of their SUMO ligase activity (their RING finger), suggesting that the role of  
5 PIAS proteins extends beyond modifying the outputs of the SUMO pathway (Johnson, 2004;  
6 Palvimo, 2007). Thus, the interaction between PIAS3 and M2PK, as shown here, could  
7 reflect either an enzyme-substrate complex with a short half life or another type of regulatory  
8 interaction. In fact, only a few proteins are quantitatively sumoylated. Instead, most targets  
9 appear to be modified to a small percentage at steady state (usually less than 5%) (Geiss-  
10 Friedlander and Melchior, 2007). It is important to consider that targets can undergo rapid  
11 cycles of modification and demodification. Although the equilibrium might lie on the side of  
12 the unmodified form, the whole pool of a given protein might be affected by sumoylation in a  
13 short window of time and there is precedence that low-level sumoylation can cause large  
14 effects (Geiss-Friedlander and Melchior, 2007). When endogenous PIAS3 was partially  
15 depleted by siRNA-mediated gene knockdown, no discernable differences in the degree of  
16 M2-PK were observed (data not shown). When the catalytically inactive mouse PIAS3 RING  
17 domain mutant PIAS3 (C299S, H301A) (Long et al., 2004) was expressed in U2-OS cells  
18 instead of wild type PIAS3, no nuclear localization of M2-PK was observed (Fig. 6). Thus,  
19 while the role of PIAS3 for sumoylation of M2-PK remains to be defined, our data suggest  
20 that PIAS3 specifically induces nuclear localization of M2-PK and this effect depends on the  
21 RING domain of PIAS3.

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44 The interaction of M2-PK with PIAS3 involves the intact carboxyl-terminal region of PIAS3  
45 from amino acid residues 383 to 619. Although the approximately 400 residues-long  
46 conserved amino-terminal region of PIAS3 is well characterized - it contains the RING finger-  
47 like zinc-binding domain mediating the SUMO-E3-ligase activity of PIAS proteins (Kotaja et  
48 al., 2002) and binds directly to Ubc9 (Kahyo et al., 2001) - the exact functions of the less  
49 conserved carboxyl-terminal region remain to be defined. This region contains an acidic  
50 motif, a serine/threonine rich region, and is involved in the interaction with specific substrate  
51 proteins (Shuai and Liu, 2005), as has been shown for some transcriptional regulators  
52 (Nakagawa and Yokosawa, 2002; Jimenez-Lara et al., 2001; Tirard et al., 2004). Although  
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3 PIAS proteins are localized predominantly in the nucleus and transcription is a major process  
4 regulated by these SUMO E3 ligases, additional processes are regulated by sumoylation and  
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7 PIAS proteins have been shown to interact with various cellular proteins, for instance  
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10 enzymes involved in DNA-replication or proteins of the cytoplasmic septin family of  
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12 cytoskeletal proteins (Johnson, 2004). Whereas SUMO conjugation is important for the  
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14 targeting of glucose transporters (GLUT4 and GLUT1) from intracellular sites to the  
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16 cytoplasmic membrane (Giorgino et al., 2000; Lalioti et al., 2002), the direct interaction of a  
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18 SUMO E3 ligase with a glycolytic enzyme, along with its subcellular relocalization, has not  
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20 previously been shown.  
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25 In this study it is shown, that overexpression of PIAS3 leads to nuclear localization of M2-PK  
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27 and the colocalization of this enzyme with PIAS3 in the nucleus of different cell types.  
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29 Little is known about M2-PK beside its important metabolic function as glycolytic enzyme in  
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31 the cytosol (reviewed in Mazurek et al., 2005). A monomeric form of M2-PK with low  
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33 enzymatic activity was previously described as a cytosolic thyroid hormone-binding protein  
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35 (Kato et al., 1989), and a subsequent study suggested that monomeric M2-PK has a role in  
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37 the regulation of thyroid hormone receptor-dependent transcription (Ashizawa, and Cheng,  
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39 1992). Previous work also provided evidence for DNA-binding and histone H1 kinase activity  
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41 of M2-PK (Guminska et al., 1988), and recent work demonstrated that M2PK can exist as a  
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43 nuclear protein. Thus, interleukin-3 induces nuclear translocation of M2-PK and this  
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45 enhances cell proliferation (Hoshino et al., 2007). Another study showed that M2-PK  
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47 translocates into the nucleus after apoptotic stimuli and contributes to the induction of  
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49 programmed cell death (Steták et al, 2007). Furthermore, a role in transcriptional regulation  
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51 was described for nuclear M2-PK (Lee et al., 2008). The present study underlines that M2-  
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53 PK exists as nuclear protein and suggests a link between the SUMO system and M2-PK.  
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57 Nuclear localization and/or functions have been previously shown for other glycolytic  
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59 enzymes (hexokinase, aldolase, glyceraldehyde 3-P dehydrogenase (GAPDH),  
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phosphoglycerate kinase and enolase) (reviewed in Sirover, 2005). In yeast, hexokinase 2

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3 acts as a nuclear factor in glucose induced transcriptional repression (Rodriguez et al.,  
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5 2001). A nuclear alternative splicing form of enolase 1 has been demonstrated to be involved  
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7 in transcriptional repression of the c-Myc gene in HeLa cells (Ghosh et al., 1999). It was  
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9 shown that GAPDH can modulate the activity of DNA-polymerase- $\alpha$ -primase complex  
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11 (Grosse et al., 1986), and nuclear GAPDH plays a role in control of apoptotic cell death in  
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13 several human cell types (Sawa et al., 1997).  
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For Peer Review

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## Legends to the figures

**Fig. 1. Identification of PIAS3 as M2-PK-binding protein. (A) (Left panel)** Expression of the baits LexA, LexA-M1-PK and LexA-M2-PK in the yeast strain EGY48 was detected by immunoblot analysis using a monoclonal anti-LexA antibody. A lysate of untransformed yeast strain EGY48 was loaded as control. **(Right panel)** Expression of the B42-HA1-PIAS3<sub>383-619</sub> prey fusion protein in EGY48 as shown by immunoblot using anti-HA1 antibodies. **(B)** Derivatives of yeast strain EGY48/pSH1834::URA3 (Mat a, his3, ura3, trp1, leu2::lexA<sub>o6</sub>-pLEU2/lexA<sub>o8</sub>-Gal1-lacZ::URA3) expressing various LexA fusion proteins from pEG202::HIS3 vectors as indicated, were transformed with the plasmid pB42-PIAS3<sub>383-619</sub>::TRP1. Transformants were selected for uracil, histidine, and tryptophane prototrophy and then streaked out onto each of 4 plates and incubated for 4 days at 30°C under the following nutrient conditions: (MM Glu +Leu) Glucose minimal medium with leucine; all strains grow. (MM Glu) Glucose minimal medium without leucine; selection for B42 fusion protein independent activation of the LexA<sub>o6</sub>-LEU2 reporter. (MM Gal/Raf) Galactose minimal medium without leucine, selecting for B42 fusion protein dependent activation of the LexA<sub>o6</sub>-LEU2 gene. (MM Gal/Raf +Leu +X-Gal) Galactose minimal medium with leucine and X-Gal, selecting for B42 fusion protein dependent activation of the LexA<sub>o8</sub>-Gal1-lacZ::URA3. Gal4 served as a positive control and LexA-APP as a negative control.

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3 **Fig. 2. M2-PK interacts with PIAS3 *in vitro*. (A) (left panel)** Purified GST or GST-M2-PK  
4 proteins immobilized on glutathione-Sepharose 4B beads were incubated with whole-cell  
5 extracts from human U-2 OS cells overexpressing the PIAS3 full length protein. The amount  
6 of PIAS3 protein that was retained after washing was determined by immunoblotting using  
7 polyclonal anti-PIAS3 antibodies (top). 5% of the lysate was loaded as input. Input of the  
8 GST proteins was controlled by Coomassie staining (bottom). **(Middle panel)** Purified GST  
9 or GST-PIAS3 proteins immobilized on glutathione-Sepharose 4B beads were incubated with  
10 whole-cell extracts from U-2 OS cells. After washing, the amount of M2-PK protein that was  
11 retained was determined by immunoblotting using a monoclonal anti-M2-PK antibody (top).  
12 5% of the lysate was loaded as input. Input of the GST proteins was controlled by  
13 Coomassie staining (bottom) **(B) Mapping** of the PIAS3/M2-PK binding domains. **(Left**  
14 **panel)** Purified GST or GST-PIAS3 protein immobilized on glutathione-Sepharose 4B beads  
15 was incubated with extracts from yeast cells expressing B42-HA-M2-PK<sub>1-531</sub> or B42-HA-M2-  
16 PK<sub>1-348</sub> fusion proteins. After washing, the amount of M2-PK protein that was retained was  
17 determined by immunoblotting using a monoclonal anti-M2-PK antibody. M2-PK-input was  
18 loaded as indicated (For input of GST proteins see Fig. 2A). **(Right panel)** Purified GST or  
19 GST-M2-PK protein immobilized on glutathione-Sepharose 4B beads was incubated with  
20 extracts from yeast cells expressing B42-HA-PIAS3<sub>383-619</sub>. After washing, the amount of the  
21 bound B42-HA-PIAS3<sub>383-619</sub> protein was determined by immunoblotting using a monoclonal  
22 anti-HA1 antibody. 5% of the input was loaded (for input of GST proteins see Fig. 2A).  
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3 **Fig. 3. M2-PK interacts with PIAS3 in U-2 OS cell extracts.** Extracts were prepared from  
4 U-2 OS cells transiently overexpressing PIAS3 and subjected to immunoprecipitation with  
5 antibodies against M2-PK, PIAS3 antibodies and control antibodies, as indicated.  
6  
7 Precipitated proteins were separated by SDS-gel electrophoresis and detected by Western  
8 blotting. Total cell lysate (100  $\mu$ g) was loaded as input control. PIAS3 was  
9 immunoprecipitated by anti-PIAS3 antibodies (left, upper panel) and M2-PK was co-  
10 immunoprecipitated (left, lower panel). M2-PK was immunoprecipitated by M2-PK specific  
11 antibodies (right, lower panel) and PIAS3 was co-immunoprecipitated (right, upper panel).  
12 The species-specific preimmune sera (IP control serum) precipitated neither M2-PK nor  
13 PIAS3.  
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3 **Fig. 4. Sumoylation of M2-PK. (A)** Detection of SUMO-1-M2-PK conjugates in U-2 OS cells.  
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5 U-2 OS cells were transiently transfected either with empty vector or with a CMV-promoter  
6 driven expression vector for PIAS3. 30 hours post-transfection, immunoprecipitations were  
7 performed as described in the sumoylation assay with M2-PK specific antibodies from PIAS3  
8 non-overexpressing (lanes 2) or PIAS3 overexpressing U-2 OS cells (lanes 3).  
9 Immunoprecipitations were also performed with an unrelated control serum as negative  
10 control (lane 1). The precipitates were separated by SDS-PAGE and Western blotted using  
11 anti-M2-PK (left panel) and anti-SUMO-1 antibodies (right panel). Input controls are shown  
12 as indicated. (lanes 4). **(B)** Decrease of higher molecular weight M2-PK conjugates in U-2  
13 OS cell lysates over time. Cells were lysed in an SDS containing buffer and the lysates  
14 placed for 6 and 18 hours at 4°C or immediately (0h) processed for Western blotting using  
15 anti-M2-PK antibodies. **(C)** The levels of endogenous and ectopically overexpressed PIAS3  
16 protein were analysed in U-2OS cells by Western blotting using anti-PIAS3 antibodies. **(D)**  
17 M2-PK-SUMO-1 conjugates were co-immunoprecipitated as in figure 4A, and the intensity of  
18 the immunoprecipitated 85 kD band from PIAS3 non-overexpressing and PIAS3  
19 overexpressing U-2 OS cells was quantified by densitometry (Statistical significance was  
20 calculated using Student's t-test, n=4, P= 0.0056 = \*\*).  
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3 **Fig. 5. Subcellular localization of PIAS3 an M2-PK.** U-2 OS or NIH3T3 cells were  
4 transiently transfected with a CMV-promoter driven expression vector for PIAS3 or the empty  
5 vector as indicated. 30 hours later, cells were fixed/permeabilized with 4%PFA/0.2%Triton-X-  
6 100 and stained with anti-PIAS3 antibodies, the DNA-stain TO-PRO-3 or anti-M2-PK  
7 antibodies as indicated and processed for indirect immunofluorescence microscopy and  
8 viewed by using a confocal scanning system. **(A)** Staining of PIAS3-overexpressing U-2 OS  
9 cells with TO-PRO-3 (left panel, red) and anti-PIAS3 antibodies (middle panel, green), Merge  
10 (right panel, yellow). **(B)** Staining of PIAS3-overexpressing U-2 OS cells with TO-PRO-3 (left  
11 panel, red) and anti-M2PK antibodies (middle panel, green), Merge (right panel, yellow). **(C)**  
12 Staining of U-2 OS / empty vector cells with TO-PRO-3 (left panel, red) and anti-M2PK  
13 antibodies (middle panel, green), Merge (right panel, yellow). **(D)** Staining of PIAS3-  
14 overexpressing U-2 OS cells with anti-M2-PK antibodies (left panel, red) and anti-PIAS3  
15 antibodies (middle panel, green), Merge (right panel, yellow). **(E)** Staining of U-2 OS / empty  
16 vector cells with anti-M2-PK antibodies (left panel, red) and anti-PIAS3 antibodies (middle  
17 panel, green), Merge (right panel, yellow). **(F)** Incubation of PIAS3-overexpressing U-2 OS  
18 cells only with the secondary anti-rabbit and anti-goat IgGs **(G)** Staining of PIAS3-  
19 overexpressing NIH3T3 cells with anti-M2-PK antibodies (left panel, red) and anti-PIAS3  
20 antibodies (middle panel, green), Merge (right panel, yellow).  
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3 **Fig. 6. Subcellular localization of endogenous M2-PK in cells overexpressing PIAS3**  
4 **wild type or the catalytically inactive RING domain mutant PIAS3 (C299S, H301A). U-2**  
5 **OS cells were transiently transfected with CMV-promoter driven expression vectors for Flag-**  
6 **mPIAS3 wild type, Flag-mPIAS3 (C299S, H301A) or the empty vector as indicated. 24 hours**  
7 **later, cells were fixed/permeabilized with 4%PFA/0.2%Triton-X-100 and stained with anti-M2-**  
8 **PK antibodies and the DNA-stain TO-PRO-3 as indicated and processed for indirect**  
9 **immunofluorescence microscopy and viewed by using a confocal scanning system. (A)**  
10 **Expression of the Flag-mPIAS3 wild type, Flag-mPIAS3 (C299S, H301A) proteins in U2-OS**  
11 **cells as shown by immunoblot using anti-Flag antibodies,  $\beta$ -actin served as input control. (B)**  
12 **Staining of Flag-mPIAS3-overexpressing U-2 OS cells with anti-M2-PK antibodies (left panel,**  
13 **green), TO-PRO-3 (middle panel, red), Merge (right panel, yellow). Cells with nuclear M2-PK**  
14 **are marked by arrows. (C) Staining of mFlag-PIAS3 (C299S, H301A) mutant-overexpressing**  
15 **U-2 OS cells with anti-M2-PK antibodies (left panel, green), TO-PRO-3 (middle panel, red),**  
16 **Merge (right panel, yellow). (D) Staining of empty vector containing U-2 OS cells with anti-**  
17 **M2-PK antibodies (left panel, green), TO-PRO-3 (middle panel, red), Merge (right panel,**  
18 **yellow).**  
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3 **Supplementary Fig. 1. Predicted sumoylation sites in M2-PK.** (Upper panel) The  
4 predicted sumoylation sites are indicated in the human M2-PK amino acid sequence. (Lower  
5 panel) M2-PK contains eight hypothetical sumoylation motifs. Most SUMO-modified proteins  
6 contain the motif YKXD/E, Y = bulky amino acid, K = lysine residue conjugated to SUMO, X=  
7 any amino acid, D or E = acidic residue. The sumoylation sites in M2-PK were predicted  
8 using [www.abgent.com/doc/sumoplot](http://www.abgent.com/doc/sumoplot).  
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