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Interleukin-6 influences stress-signalling by reducing the expression of the mTOR-Inhibitor REDD1 in a STAT3-dependent manner

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Abstract:

Interleukin 6 is a pleiotropic cytokine and a strong activator of Mammalian Target of Rapamycin (mTOR). In contrast, mTOR activity is negatively regulated by Regulated in Development and DNA Damage Responses 1 (REDD1). Expression of REDD1 is induced by cellular stressors such as glucocorticoids and DNA damaging agents. We show that the expression of basal as well as stress-induced REDD1 is reduced by IL-6. The reduction of REDD1 expression by IL-6 is independent of proteasomal or caspase-mediated degradation of REDD1 protein. Instead, induction of REDD1 mRNA is reduced by IL-6. The regulation of REDD1 expression by interleukin 6 (IL-6) is independent of Phosphatidylinositide-3-Kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK) signalling but depends on the expression and activation of Signal Transducer and Activator of Transcription 3 (STAT3). Furthermore, the reduction of basal REDD1 expression by IL-6 correlates with IL-6-induced activation of mTOR signalling. Inhibition of STAT3 activation blocks IL-6-induced mTOR activation. In summary, we present a novel STAT3-dependent mechanism of both IL-6-induced activation of mTOR and IL-6-dependent reversion of stress-induced inhibition of mTOR activity.

Keywords

Interleukin 6, stress signalling, REDD1, STAT3, mTOR

Footnote

Abbreviations: Hypoxia-inducible Factor (HIF), Interleukin 6 (IL-6), Insulin Receptor Substrate 1 (IRS1), Januskinase (Jak), Mammalian Target of Rapamycin (mTOR), Mouse Embryonic Fibroblast (MEF), Nuclear Factor kappa B (NF-κB), n.s. (non-significant), Mitogen-Activated Protein Kinase (MAPK), Oncocstatin M (OSM), P70 S6 kinase (P70 S6K), Phosphatidylinositide-3-kinase (PI3K), Protein Phosphatase 2A (PP2A), Pyrrolidine Dithiocarbamate (PDTC), Ras Homolog Enriched in Brain (RHEB), Regulated in Development and DNA Damage Responses 1 (REDD1), Reactive Oxygen Species (ROS), S6 Ribosomal Binding Protein (S6RBP), Suppressor of Cytokine Signalling 3 (SOCS3), Signal Transducer and Activator of Transcription 3 (STAT3), Tuberous Sclerosis Complex (TSC)

Highlights

- IL-6 interferes in stress signalling by reducing expression of the mTOR inhibitor REDD1
- Reduction of REDD1 occurs on the level of mRNA induction
- Reduction of REDD1 is independent of PI3K and MAPK but depends on STAT3 activation
- Reduction of REDD1 contributes to IL-6-induced activation of mTOR signalling

1. Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine that orchestrates a plethora of pro- and anti-inflammatory functions. By binding to a receptor complex consisting of IL-6Rα and the signal transducing subunit gp130 IL-6 induces the activation of Januskinases (Jak). Activated Jak in turn initiate the Jak/Signal Transducer and Activator of Transcription (STAT), Mitogen Activated Protein Kinase (MAPK) and Phosphoinositide 3-kinase (PI3K) pathways [1]. Additionally IL-6 activates Mammalian Target of Rapamycin (mTOR) [2]. The mechanisms of activation of Jak/STAT, MAPK and PI3K pathways have been analysed in detail [1, 3]. However, the molecular mechanisms leading to the activation of mTOR in response to IL-6 are not yet understood completely.

mTOR is a master regulator of cellular growth and metabolism. Canonical activation of mTOR is driven by the PI3K-dependent phosphorylation of the Tuberous Sclerosis Complex (TSC). Phosphorylation of the TSC complex results in inactivation of the GTPase function of the small GTPase Ras Homolog Enriched in Brain (RHEB). GTP-bound RHEB stimulates mTOR activity. Activated mTOR phosphorylates downstream effector proteins such as members of the eukaryotic translational initiation machine and the P70 S6 kinase (P70 S6K) which phosphorylates S6 Ribosomal Binding Protein (S6RBP) [4]. mTOR activation by IL-6 is more complex and involves more than one IL-6-induced signalling pathway. IL-6 has the ability to canonically activate mTOR by activation of PI3K. However, also the inhibition of MAPK signalling reduces IL-6-induced phosphorylation of P70 S6K [2].

In response to cellular stress mTOR function is inhibited to regulate cellular energy balance. The control of energy supply and consumption is part of the stress response and is intensively studied in response to the stress hormone cortisol [5]. One important mediator of the cellular stress response is the mTOR inhibitor Regulated in Development and DNA Damage Responses 1 (REDD1, DIG2, DDIT4, RTP801). Several stressors such as energy depletion [6], ER stress [7, 8], hypoxia [9, 10], DNA damage by irradiation [11] and chemicals [12] induce the expression of REDD1. Additionally REDD1 expression is increased by pharmacological derivatives of cortisol such as dexamethasone [8, 13] and by insulin [14].

REDD1 has no known structural or functional domain and most presumably inhibits mTOR by interacting with other proteins involved in mTOR signalling [15] such as 14-3-3 proteins which regulate the TSC-complex [16] or Protein Phosphatase 2A (PP2A) which dephosphorylates and thus inactivates PI3K [17]. REDD1-mRNA and protein-turnover are heavily regulated by several miRNAs [18, 19] and by ubiquitin-dependent proteasomal degradation [20] or caspase-mediated cleavage [21], respectively.

Dysregulation of REDD1 expression and thereby disturbed stress signalling has tremendous effects on cellular homeostasis. Increased REDD1 expression in neurodegenerative diseases such as Parkinson´s and Alzheimer´s disease leads to permanent inhibition of mTOR and thereby cellular death [22, 23]. On the contrary, reduced REDD1 expression and constitutive mTOR signalling is found in cancer patients [16, 18].

The opposed regulation of mTOR signalling by cellular stressors and inflammatory mediators such as IL-6 raises expectations of complex cross-talk events between stress- and cytokine-induced signalling. The multifaceted regulation of REDD1 expression and function makes REDD1 an important target for this cross-talk. Here, we demonstrate that IL-6 reduces basal as well as stress-induced REDD1 expres-

sion in a STAT3-dependent manner thereby causing reversion of stress-induced signalling and activation of mTOR.

2. Materials and Methods

2.1 Cloning

REDD1 cDNA was amplified from pFN21A-REDD1 (Promega, Madison, WI, USA) with the following primers fw: 5‘- ACTTATGGTACCATGCCTAGCCTTTGGG-3‘; rv: 5‘-T TAAAGGATCCTTAACACTCCTCAATGG G-3’ (MWG-Biotech, Ebersberg, Germany) and cloned into pcDNA3 (Thermo Fisher Scientific, Waltham, MA, USA) using BamHI and KpnI (Cell Signalling Technology, Frankfurt/Main, Germany). STAT3 cDNA was amplified from pcDNA5-STAT3-YFP (kindly provided by Prof. G. Müller-Newen) with the following primers fw: 5‘-GCGCGGATCCATGGCTCAGTG-3‘; rv: 5‘-TATACTCGAGTTACATGGGGGAG-3’ (MWG-Biotech) and cloned into pcDNA3 using BamHI and XhoI (Cell Signalling Technology).

2.2 Cell Culture

HepG2 (DSMZ, Braunschweig, Germany) were grown in DMEM+F12 (Thermo Fisher Scientific) supplemented with 10 % FCS, streptomycin (100 mg/ml) and penicillin (100 mg/ml) at 37°C in a water saturated atmosphere containing 5 % CO_2. Prior to stimulation cells were starved overnight in medium without FCS and phenol red. Cells were treated with 10 ng/ml IL-6 (Conaris, Kiel, Germany), 10 ng/ml OSM (Peprotech, Hamburg, Germany), 1 µM dexamethasone (Sigma Aldrich, St. Louis, MO, USA), 500 ng/ml insulin (Sigma Aldrich), 0.5 µM campthotecin (Sigma Aldrich), 10 µM MG-115 (Diagonal, Münster, Germany), 50 µg/ml cycloheximide (Sigma Aldrich), 4 µM actinomycin D (VWR, Darmstadt, Germany), 10 µM U0126 (Promega), 40 µM LY294002 (VWR) or 20 µM Stattic (VWR) as indicated.

2.3 γ-Irradiation

HepG2 cells were exposed to ionizing radiation in a Biobeam GM 2000 (Gamma Medical Service, Leipzig, Germany) with ^{137}Cs as radioactive isotope and a dose rate of approximately 3 Gy/min.

2.4 Transfection

Cells were transfected with ON-TARGETplus SMART pool siRNA against human Dicer 1 (#23405), human STAT3 (#6774) or control siRNA (GE Healthcare Life Sciences, Chalfont St Giles, UK) using Dharmafect 4 according to manufacturer’s instructions. Cells were harvested 72 h after transfection.

Cells were transfected with pcDNA3 expression vectors for REDD1 and STAT3 respectively using electroporation with nucleofector technology (Lonza, Basel, Switzerland) according to manufacturer`s instructions. Cells were harvested 24 h after transfection.

2.5 Quantitative RT PCR

Total RNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. 1 µg of RNA was reverse transcribed into cDNA with Omniscript (Qiagen) using random hexameric primers according to manufacturer’s instructions. Taqman gene expression assays for human REDD1 (Hs01111686_g1) and human HPRT (Hs99999909_m1) were obtained from Thermo Fisher Scientific and PCR was performed using qPCR Mastermix plus (Eurogentec, Cologne, Germany). The PCR reaction was performed in a final volume of 20 µl containing 2 µl cDNA and 1 µl Taqman

gene expression assay solution. Following a 15 min denaturing step at 94°C amplification was performed in 40 cycles (15 s at 94°C, 60 s at 60°C, 30 s at 70°C) on a Rotorgene (Qiagen). Quantification of gene expression was calculated as described by Pfaffl et al. [24].

2.6 Western Blotting

For the isolation of cellular proteins cells were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 % NP-40, 15 % Glycerol, supplemented with 10 µg/ml of each aprotinin, leupeptin and pepstatin as well as 0.8 µM Pefabloc (Roche, Mannheim, Germany), 1 mM NaF and 1 mM Na_3VO_4). The protein concentration of the lysates was determined using Biorad Protein Assay (Biorad, Munich, Germany). Proteins were separated by SDS-PAGE and transferred to a polyvinylidenedifluorid membrane. Antigens were detected by incubation with specific primary antibodies (1:1000) followed by incubation with horseradish-peroxidase-coupled secondary antibodies (1:7500) (DAKO, Hamburg, Germany). Detection occurred via an enhanced chemoluminescence kit (Millipore Corporation, Darmstadt, Germany). List of primary antibodies: Dicer (#3363), phosphorylated ERK1/2 (#4370), ERK1/2 (#4695), phosphorylated STAT3 (#9145), phosphorylated S6RBP (#2211), S6RBP (#2217) (Cell Signalling Technology, Frankfurt/Main, Germany); STAT3 (#482) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); HSC70 (1B5) (Farmingdale, NY, USA); Tubulin (B-5-1-2) (Sigma Aldrich) and REDD1 (#10638) (Proteintech, Chicago, Il, USA). Western blots were quantified using Multi Gauge software (Fujifilm Life Sciences, Düsseldorf, Germany).

2.7 Data Analysis

Experiments were performed in n = 3 independent biological replicates. Bar diagrams display means ± standard deviation of these independent experiments. Statistical analysis with rANOVA or student´s t-test with * $p < 0,1$; ** $p < 0,01$; *** $p < 0,001$ was done using GraphPad InStat (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1 IL-6 reduces stress-induced REDD1 expression

The mTOR-inhibitor REDD1 is induced by different stress-stimuli. To test whether IL-6 counteracts stress-induced REDD1 protein expression human hepatoma HepG2 cells were stressed by irradiation (Fig. 1A, Suppl. Fig. 1A), by insulin treatment (Fig. 1B, Suppl. Fig. 1B), by the topoisomerase inhibitor campthotecin (Fig. 1C, Suppl. Fig. 1C) or by the glucocorticoid derivate dexamethasone (Fig. 1D, Suppl. Fig. 1D) to induce REDD1-expression. For comparison cells were left untreated (1st lanes). In addition to stress treatment cells were stimulated for 75 min with IL-6 (last lanes) prior to analysis of REDD1-expression by Western blotting. STAT3-activation was monitored to validate IL-6 signalling (Suppl. Fig 2). Figure 1 and Suppl. Fig 1 demonstrate that treatment of HepG2 cells with IL-6 significantly reduces stress-induced REDD1 protein expression independent of the stress source. This effect of IL-6 is specific for REDD1 as REDD2 expression is not affected by IL-6 (Suppl. Fig. 3).

3.1 IL-6 does not affect REDD1 protein turnover

We first analysed the time response of IL-6-dependent reduction of dexamethasone-induced REDD1 protein expression. Figure 2A shows inhibition of REDD1 expression after 40 minutes of IL-6 treatment (1st panel). Interestingly, the peak of the transient STAT3 activation precedes REDD1 reduction (Suppl. Fig. 4A and 4B).

To test whether IL-6 interferes in REDD1 expression through the activation of proteasomal degradation we monitored REDD1 protein expression in the presence of the proteasomal inhibitor MG-115. Supplementary figure 5 shows that DMSO the solvent for MG-115 (and for other inhibitors used in this study) does not influence the stress-induced expression and IL-6-induced reduction of REDD1 protein. As shown in figure 2B and suppl. fig. 6, inhibition of proteasomal degradation strongly increases basal REDD1 expression (compare 1st and 5th lane, panel 1), demonstrating high basal turnover of REDD1 protein. However, the reduction of REDD1 protein expression by IL-6 was not impaired in the presence of MG-115 (compare 6th with 8th lane and 5th with 7th lane) indicating that IL-6 counteracts REDD1 expression independent of proteasomal protein degradation. Puissant et al. describe caspase-3 mediated cleavage of REDD1 [21]. However, this mechanism of REDD1 degradation was excluded as IL-6 treatment did not result in cleaved REDD-1 fragments (Suppl. Fig. 7). Additionally, we compared the decay of REDD1 protein in the absence and presence of IL-6 (Fig. 2C, Suppl. Fig. 8). To do so we blocked protein synthesis by cycloheximide treatment. Although REDD1-expression was reduced in the presence of IL-6 (compare 1st and 10th lane) the decay of REDD1 protein was not changed by IL-6.

Finally, we analysed the effect of IL-6 on exogenously expressed REDD1 protein. First, we analysed the decay of exogenously expressed REDD1 protein in the presence of cycloheximide and confirmed fast degradation of REDD1 (Fig. 2D, Suppl. Fig. 9). Notably, IL-6 had no effect on the amount of exogenously expressed REDD1 as REDD1 protein expression did not change significantly in the presence of IL-6 for up to 90 minutes (Fig. 2E, Suppl. Fig. 10).

In summary, these results demonstrate that IL-6 does not affect REDD1 protein expression by influencing REDD1 protein turnover.

3.2 IL-6 reduces REDD1 mRNA induction

As IL-6 does not reduce REDD1 protein expression by affecting REDD1 protein turnover we tested whether IL-6 influences REDD1 mRNA expression. Real-time PCR analyses revealed that dexamethasone increases not only REDD1 protein but also REDD1 mRNA expression. Interestingly, IL-6 reduces both basal REDD1 mRNA and dexamethasone-induced REDD1 mRNA expression significantly (Fig. 3A). REDD1 mRNA is subject to miRNA dependent regulation [18, 19]. miRNA processing depends on the RNAse III-type endonuclease Dicer [25]. To test whether miRNA-dependent regulation of gene expression is involved in IL-6-dependent repression of REDD1-mRNA we depleted Dicer to impair miRNA processing by an siRNA approach. To examine whether knock-down of Dicer reduces the processing of miRNAs we analysed exemplarily the expression of miR-18a_2 which is constitutively expressed in liver cells [26]. Suppl. Fig. 11 shows that knock-down of Dicer indeed significantly reduces the amount of mature miR-18a_2 compared to control siRNA treated cells. However, the knock-down of Dicer expression had no significant effect on IL-6-mediated repression of REDD1 mRNA and thus no evidence for miRNA-mediated regulation of REDD1 mRNA is given (Fig. 3B).

Finally, we compared the decay of REDD1 mRNA in the presence and absence of IL-6. Ongoing transcriptional activity was blocked by actinomycin D and REDD1 mRNA was analysed for up to 150 min by real-time PCR. Figure 3C shows that IL-6 does not affect REDD1 mRNA turnover to reduce REDD1 protein expression. However, IL-6 reduces the steady state amount of REDD1 mRNA (Fig. 3A) indicating that IL-6 influences REDD1 mRNA induction.

3.3 STAT3 expression and activation is crucial for IL-6-dependent reduction of REDD1 expression

To examine whether STAT3 activation contributes to IL-6-mediated reduction of REDD1 expression we utilized the STAT3 inhibitor Stattic which impairs IL-6-dependent phosphorylation of STAT3 (Fig. 4A, compare 3rd and 7th lane). Interestingly, and similar to MG-115 (Fig. 2B) Stattic increases basal REDD1 protein expression. However, Stattic treatment (in contrast to MG-115 treatment) abolished IL-6-dependent reduction of REDD1 protein expression (compare lanes 1-4 with 5-8). Beside REDD1 protein also REDD1 mRNA expression was not affected by IL-6 in the presence of Stattic (Fig. 4B, black bars). In accordance with these results overexpression of STAT3 reduces basal REDD1 protein expression (Fig. 4C), whereas knock-down of STAT3 by siRNA increases REDD1 expression (Fig. 4D). Similarly, enhanced REDD1 expression was observed in STAT3-deficient MEF cells but not in corresponding STAT3 floxed MEF cells or STAT3-deficient cells reconstituted with STAT3. (Suppl. Fig. 12). These data clearly indicate a crucial role of STAT3 expression and activation in reduction of REDD1 expression by IL-6.

3.4 Reduction of REDD1 expression is independent of IL-6-induced MAPK and PI3K activation

Next we asked whether IL-6-induced activation of the MAPK- and PI3K-cascade is also involved in the IL-6-dependent reduction of REDD1. Therefore, we analysed REDD1 mRNA (Fig. 5A) and protein expression (Fig. 5B and C) in the presence or absence of the MEK-inhibitor U0126 and the PI3K inhibitor LY294002, respectively. To monitor efficacy of the inhibitors phosphorylation of the downstream targets ERK1/2 (for U0126) (Fig. 5B) and S6RBP (for LY294002) (Fig. 5C) was analysed. As demonstrated in Fig. 5 neither of both inhibitors interferes in IL-6-dependent repression of REDD1 mRNA (Fig. 5A) or REDD1 protein expression (Fig 5B and C). These data indicate that activation of the MAPK- and the PI3K-pathway by IL-6 does not influence REDD1 expression.

3.5 IL-6-induced reduction of REDD1 correlates with the activation of mTOR signalling by IL-6

In Fig. 5C (3rd lane, panel 2) we demonstrate IL-6-dependent phosphorylation of the mTOR target S6RBP. However, it is still not fully understood which pathways contribute to IL-6 dependent activation of mTOR. Based on previous data [2] IL-6-induced MAPK activation contributes to mTOR signalling in myeloma cells. Thus, we tested whether inhibition of the MAPK cascade by the MEK inhibitor U0126 also affects phosphorylation of the mTOR substrate S6RBP in HepG2 cells. As demonstrated in Fig. 6A inhibition of the MAPK cascade also abolishes IL-6-dependent phosphorylation of S6RBP in HepG2 cells.

Based on our observation that IL-6 reduces basal REDD1 expression (Fig. 5A) we developed the hypothesis that this reduction of REDD1 contributes to the activation of mTOR. In line with this idea and based on the fact that REDD1 repression requires STAT3 activation (Fig. 4) we tested whether STAT3 activation is also required for IL-6-induced S6RBP phosphorylation. Figure 6B demonstrates that in the presence of the STAT3 inhibitor Stattic IL-6 fails to induce phosphorylation of S6RBP (compare 3rd and 6th lane, panel 1). This supports the idea that IL-6-induced STAT3 activation is crucial for mTOR activation and subsequent S6RBP phosphorylation.

To further test our hypothesis that IL-6-induced S6RBP phosphorylation is caused by reduced REDD1 expression we compared the kinetics of IL-6-dependent repression of REDD1 protein expression and IL-6-dependent phosphorylation of S6RBP (Fig. 6C). Whereas IL-6-induced STAT3 and MAPK activation peak within less than one hour [27, 28] IL-6-induced mTOR activation is delayed and reaches a maximum at 75 min (Fig 6C, compare panels 1 and 2 and quantification of data). In parallel, basal REDD1 expression is reduced by IL-6 within the first hour of stimulation and remains low for up to

120 min. Thus, in line with our hypothesis that IL-6-induced REDD1 reduction causes mTOR activation REDD1 protein expression and phosphorylation of S6RBP correlate inversely.

3.6 Repression of REDD1 expression is not limited to IL-6

Due to the fact of the crucial role of STAT3 activation for the repression of REDD1 we tested whether Oncostatin M (OSM), another STAT3-activating cytokine, also represses stress-induced REDD1 expression. As shown in Fig.1 for IL-6 also OSM induces STAT3 phosphorylation and counteracts REDD1 expression in response to irradiation, insulin, campthotecin and dexamethasone treatment (Fig.7). These observations further support our hypothesis that STAT3 activating cytokines such as IL-6 and OSM regulate mTOR signalling by counteracting the expression of the mTOR inhibitor REDD1.

4. Discussion

In the presented study, we investigated the mechanism of IL-6-induced reduction of the mTOR inhibitor REDD1. In summary IL-6 and other STAT3 activating cytokines reduce the induction of REDD1 mRNA in a STAT3-dependent manner independent of PI3K and MAPK signalling (Fig. 8).

Endogenous as well as exogenous REDD1 protein is rapidly degraded within less than 15 min (Fig.2 C and D) as it has also been shown by Kimball et al. [29]. Katiyar et al. describe ubiquitination-dependent proteasomal degradation of REDD1 [20]. This finding is supported by the fact that basal REDD1 expression is strongly increased by treatment with the proteasomal inhibitor MG-115 (Fig. 2B). As the reduction of REDD1 by IL-6 requires 40 min (Fig. 2A) we hypothesized that induction or activation of an ubiquitin ligase might reduce REDD1 expression. The IL-6-induced suppressor of cytokine signalling 3 (SOCS3) promotes the ubiquitination of target proteins via binding to elongin BC and cullin5 [30]. In accordance with the timespan needed to reduce REDD1 expression maximal IL-6-induced SOCS3 expression occurs within 30 to 60 min [31]. However, blocking of proteasomal degradation does not influence IL-6-induced reduction of REDD1 protein (Fig. 2B). Additionally, REDD1 was not found to be a target of the SOCS3 E3-ligase complex in a recent unbiased screen for SOCS3 targets [32]. In summary, REDD1 expression per se is regulated by ubiquitin-dependent proteasomal degradation but the IL-6-dependent reduction of REDD1 is independent of protein degradation or other mechanisms influencing protein stability (Fig. 2B,C).

Yet REDD1 expression is regulated on mRNA level (Fig. 3A). The decay of REDD1 mRNA (Fig 3B) is in accordance with the half-life of approximately 30 min described by Mata et al. [33]. However, IL-6 does not decrease REDD1 mRNA stability (Fig. 3C) whereas it decreases mRNA amount (Fig. 3A). This observation points to an IL-6-dependent regulation of REDD1 mRNA induction. We show that this regulation is STAT3 dependent (Fig. 4). Interestingly, binding of STAT3 to putative REDD1 regulatory DNA domains has been shown by CHIP-sequencing [34] giving further evidence for a direct regulation of REDD1 expression by STAT3.

Note, that the basal expression of the REDD1 homologue REDD2 (DDIT4L, RTP801L) is not reduced by IL-6 (supp. Fig S3). REDD2 protein shares 33% sequence identity with REDD1 [35]. However, their promoter regions are dissimilar so that differential regulation is expected. REDD1 mRNA expression is induced by several transcription factors such as glucocorticoid receptor [8], ATF4 [36], hypoxia-inducible factor (HIF) [37], and p53 [12]. Based on the different transcription factors involved in induction of REDD1 and the fact that inhibition of STAT3 also increases basal REDD1 expression (Fig. 4)

it is unlikely that the reduction of REDD1 is caused by direct interaction of STAT3 with other transcription factors.

We postulate, that IL-6-dependent REDD1 reduction is a new STAT3-dependent pathway of mTOR activation. This finding is underlined by the observation that reduction of STAT3 phosphorylation by pyrrolidine dithiocarbamate (PDTC) correlates with reduction of mTOR signalling [38]. Of note, PDTC does not specifically inhibit STAT3 activation as it also inhibits e.g. nuclear factor kappa B (NF-κB) signalling [39].

Note, that the STAT3-dependent activation of mTOR signalling is not the only pathway that leads to activation of mTOR in response to IL-6. Canonical PI3K-dependent activation of mTOR-signalling in response to IL-6 depends on the activation of PI3K (Fig. 5C) through direct or indirect recruitment to gp130 [40, 41] or Gab1 [42]. The exact role of MAPK activation in IL-6-induced mTOR activation is still unclear. However, the influence of MAPK activation on S6K activation that is shown in this study (Fig. 6A) has also been demonstrated by Shi et al. [2].

The contribution of IL-6-induced mTOR activation to the functions of IL-6 is wide-ranging and mainly related to disease development. mTOR is essential for IL-6-induced tumor growth [43], it mediates IL-6-induced hepatic insulin resistance [44] and contributes to the development of non-alcoholic fatty liver disease in inflammatory conditions [45]. Interestingly, mTOR activity triggers shedding of the IL-6Rα, thereby increasing pro-inflammatory IL-6 trans-signalling hence building a positive feedback loop [46].

Induction of REDD1 is an important mechanism to overcome stress-induced impairment of cellular functions. REDD1 expression enables stress-induced autophagy by inhibition of mTOR signalling [11, 47]. Autophagy contributes to stress-clearance by controlled elimination of damaged or harmful cellular components [48]. Additionally, REDD1 sensitizes tumor cells to apoptosis and fosters tumor clearance [49]. Mitochondrial REDD1 reduces production of reactive oxygen species (ROS) and thus decreases tumorigenesis [37]. The IL-6-induced reduction of REDD1 expression reverses these effects and thereby potentially perturbs cellular stress coping. In line with this REDD1 deficient cells are strongly tumorigenic [37] and proliferate anchorage-independent [16]. Additionally, reduction of REDD1 expression has been shown to contribute to hepatocarcinogenesis [18]. Therefore, reduction of REDD1 by IL-6 could contribute to the development of proliferative diseases in inflammatory conditions. In line with this hypothesis activation of mTOR is required for inflammation-associated development of gastrointestinal tumors [43]. However, also increased REDD1 expression has been linked to the development of ovarian [50] and prostate cancer by desensitizing cells to apoptotic stimuli [51]. Thus, the exact influence of IL-6-induced reduction of REDD1 remains to be analysed.

Treatment with IL-6 reduces the expression of basal as well as DNA-damage-, insulin- and dexamethasone-induced REDD1 in a STAT3-dependent manner. As mentioned above Song et al. show that PDTC reduces IL-6-induced mTOR signalling by induction of REDD1 [38]. PDTC-induced REDD1 expression is not reduced by IL-6. However, in contrast to stressors analysed in our study PDTC also blocks IL-6-induced activation of STAT3. Consequently, PDTC-induced REDD1 cannot be downregulated by IL-6.

mTOR is an important negative regulator of insulin signalling by phosphorylating an inhibitory serine-residue in insulin receptor substrate 1 (IRS1) [52]. Insulin-dependent REDD1 expression reduces this inhibitory phosphorylation and enables proper insulin signalling via IRS-1 [14]. IL-6 is known to be

implicated in development of insulin resistance. So far this effect was primarily attributed to the IL-6-dependet expression of SOCS3 that blocks insulin receptor activity [53]. The IL-6-dependent reduction of insulin-induced REDD1-expression presented here (Fig. 1B) might additionally counteract insulin signalling and contribute to insulin resistance.

Dexamethasone was one of the first inducer of REDD1 described. The induction of REDD1 by dexamethasone explains glucocorticoid mediated apoptosis in T cells [8]. The reduction of REDD1 by IL-6 might act as a pro-survival signal to prevent dexamethasone-induced apoptosis. Indeed, IL-6 is known to mediate anti-apoptotic signals in T cells [54]. Interestingly, the anti-apoptotic function of IL-6 in T cells [54, 55] as well as the reduction of REDD1 expression depends on STAT3 (Fig. 4). Thus, relevance of REDD1 regulation in apoptosis control is likely. The cross-talk of anti-inflammatory glucocorticoids and IL-6 is not restricted to T cells but also very prominent in hepatocytes [46]. Whereas the mechanisms of glucocorticoid-induced increase in IL-6-induced Jak/STAT signalling have been analysed extensively [56] the reduction of REDD1 by IL-6 will shift focus on the mutual regulation of mTOR activity.

Beside IL-6 also the pro-inflammatory cytokine OSM decreases REDD1 expression (Fig.7) indicating that regulation of REDD1 primarily depends on the activation of STAT3. Stimulation of macrophages with LPS provokes secretion of IL-6 and subsequent autocrine stimulation of Jak/STAT signalling [57]. Also LPS induces REDD1 expression both in short and long term experiments [58]. The complexity of crosstalk scenarios including REDD1 becomes even more apparent when taking into account that REDD1 activity is not only controlled by regulation of REDD1 expression but also by sequestering REDD1 to certain cellular compartments [59]. These observations indicate that REDD1 expression is regulated in composite crosstalk scenarios that will be subject to future investigations.

5. Conclusion

In summary, we show that IL-6 reduces the expression of the mTOR inhibitor REDD1. This reduction is independent of post-translational modification of REDD1 protein but occurs on the level of REDD1 mRNA induction. The reduction of REDD1 by IL-6 presents a novel STAT3-dependent mechanism of both IL-6-induced activation of mTOR and IL-6-dependent reversion of stress-induced inhibition of mTOR that might contribute to the development of proliferative diseases in inflammatory conditions.

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Figure Captions

Figure 1: IL-6-reduces stress-induced REDD1 protein expression

HepG2 cells were stimulated with IL-6 with or without **A)** ionizing irradiation, **B)** insulin, **C)** campthotecin and **D)** dexamethasone for the indicated times. STAT3 phosphorylation as well as REDD1, STAT3 and HSC70 protein expression was evaluated by Western blotting. Representative results of n = 3 independent experiments are shown. Quantification and statistical analysis of the data is shown in suppl. fig. 1 A-D and 2 A-D.

Figure 2: IL-6-reduced REDD1 expression without influencing protein stability

A) HepG2 cells were stimulated with dexamethasone for 90 min and subsequently treated with IL-6 for the indicated times. **B)** HepG2 cells were treated for 30 min with MG-115 before stimulation with dexamethasone for 90 min and treatment with IL-6 for 75 min. **C)** HepG2 cells were stimulated with IL-6 for 45 min and subsequently treated with cycloheximide with or without IL-6 for the indicated times. **D, E**) HepG2 cells were transfected with pcDNA3-REDD1. 24 h after transfection cells were treated with cycloheximide (**D**) or IL-6 (**E**) for the indicated times. STAT3 phosphorylation as well as REDD1, STAT3 and HSC70 protein expression was evaluated by Western blotting. Representative results of n = 3 independent experiments are shown. Quantification and statistical analysis of the data is shown in suppl. fig. S4, S6, S8, S9 and S10.

Figure 3: IL-6 reduces REDD1 mRNA induction

A) HepG2 cells were pretreated with Dex for 30 min and subsequently additionally stimulated with IL-6 for 75 min or treated with Dex or IL-6 alone. The expression of REDD1 mRNA was analysed using qRT-PCR. Maximal expression of REDD1 mRNA was set to 100 %. Data are given as mean of three independent experiments ± SD. rANOVA: ** $p < 0,01$; *** $p < 0,001$ **B)** HepG2 cells were transfected with Ctr.-siRNA or Dicer-siRNA. 72 h after transfection cells were pretreated with Dex for 30 min and subsequently stimulated with IL-6 for 75 min or treated with Dex or IL-6 alone. Dicer and HSC70 protein expression were evaluated by Western blotting. The expression of REDD1 mRNA was analysed using qRT-PCR. Maximal expression of REDD1 mRNA was set to 100 %. Data are given as mean of three independent experiments ± SD. rANOVA: n.s. **C)** HepG2 cells were stimulated with IL-6 for 75 min and subsequently treated with actinomycin D with or without IL-6 for the indicated times. The expression of REDD1 mRNA was analysed using qRT-PCR. Maximal expression of REDD1 mRNA was set to 100 %. Data are given as mean of three independent experiments ± SD.

Figure 4: Reduction of REDD1 depends on STAT3

HepG2 cells were pretreated for 30 min with Stattic before additional stimulation with dexamethasone for 90 min and with IL-6 for 75 min. **A)** STAT3 phosphorylation as well as REDD1, STAT3 and HSC70 protein expression was evaluated by Western blotting. A representative result of n = 3 independent experiments is shown. **B)** The expression of REDD1 mRNA was analysed using qRT-PCR. Maximal expression of REDD1 mRNA was set to 100 %. Data are given as mean of three independent experiments ± SD. rANOVA: n.s. C) HepG2 cells were transfected with pcDNA-STAT3. 24 h after transfection cells were harvested. STAT3, REDD1 and HSC70 protein expression was evaluated by Western blotting. A representative result of n = 3 independent experiments is shown. D) HepG2 cells were transfected with Ctr.-siRNA or STAT3-siRNA. 72 h after transfection cells were

harvested. STAT3, REDD1 and HSC70 protein expression was evaluated by Western blotting. A representative result of n = 3 independent experiments is shown.

Figure 5: Reduction of REDD1 is independent of activation MAPK and PI3K pathways

HepG2 cells were treated for 30 min with U0126 or LY294002 before stimulation with dexamethasone for 90 min and treatment with IL-6 for 75 min. **A)** The expression of REDD1 mRNA was analysed using qRT-PCR. Maximal expression of REDD1 mRNA was set to 100 %. Data are given as mean of three independent experiments ± SD. **B, C)** ERK1/2, STAT3 and S6RBP phosphorylation as well as ERK1/2, HSC70, REDD1, STAT3 and S6RBP protein expression were evaluated by Western blotting. Representative results of n = 3 independent experiments are shown.

Figure 6: IL-6-induced mTOR activation depends on the activation of MAPK, PI3K and STAT3 pathways

A, B) HepG2 cells were treated for 30 min with U0126 or Stattic before stimulation with IL-6 for the indicated times. S6RBP phosphorylation and ERK1/2 or STAT3 phosphorylation as well as S6RBP, HSC70, ERK1/2 or STAT3 protein expression were evaluated by Western blotting. Representative results of n = 3 independent experiments are shown. **C)** HepG2 cells were stimulated with IL-6 for the indicated times. S6RBP phosphorylation as well as S6RBP, REDD1, and tubulin protein expression were evaluated by Western blotting. A representative result of n = 3 independent experiments is shown. For image quantification signals of REDD1, phosphorylated S6RBP, and tubulin were analysed. The diagram shows the ratio of REDD1 to tubulin as well as the ratio of phosphorylated S6RBP to tubulin for each timepoint. Basal expression of REDD1 and maximal phosphorylation of S6RBP were set to 100 %. Data are given as mean of three independent experiments ± SD.

Figure 7: STAT3 dependent reduction of REDD1 does not depend on IL-6

HepG2 cells were stimulated with OSM with or without **A)** ionizing irradiation, **B)** insulin, **C)** campthotecin and **D)** dexamethasone for the indicated times. STAT3 phosphorylation as well as REDD1, STAT3 and HSC70 protein expression was evaluated by Western blotting. Representative results of n = 3 independent experiments are shown.

Figure 8: Regulation of REDD1 expression and mTOR activation by IL-6

IL-6-induced activation of mTOR is blocked by inhibition of PI3K, MAPK and STAT3 pathways. Additionally, IL-6 interferes with mTOR signalling by reducing basal and stress-induced REDD1 mRNA expression through a STAT3-dependent mechanism.

Supplementary Figure Captions

Figure S1: IL-6-reduces stress-induced REDD1 protein expression

For quantification signals of REDD1 and HSC70 as shown in Fig. 1 A - D were analysed. The diagrams show the ratio of REDD1 to HSC70. Maximal expression of REDD1 was set to 100 %. Data are given as mean of three independent experiments ± SD. rANOVA: n.s.; * $p < 0,1$; ** $p < 0,01$; *** $p < 0,001$

Figure S2: IL-6-induces STAT3 phosphorylation independent of stress signalling

For quantification signals of phosphorylated and total STAT3 as shown in Fig. 1 A - D were analysed. The diagrams show the ratio of phosphorylated STAT3 to STAT3. IL-6-induced phosphorylation of STAT3 was set to 100 %. Data are given as mean of three independent experiments ± SD. rANOVA: n.s.; * $p < 0,1$; ** $p < 0,01$

Figure S3: REDD2 expression is not reduced by IL-6

HepG2 cells were stimulated with IL-6 for the indicated times. STAT3 phosphorylation as well as REDD2 (#160719) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), STAT3, and HSC70 protein expression was evaluated by Western blotting. A representative result of $n = 3$ independent experiments is shown.

Figure S4: IL-6 reduces REDD1 expression within 40 min

For image quantification signals of REDD1, HSC70 as well as phosphorylated and total STAT3 as shown in Fig. 2A were analysed. **A)** shows the ratio of REDD1 to HSC70 and **B)** of phosphorylated STAT3 to STAT3. Dex-induced expression of REDD1 as well as maximal phosphorylation of STAT3 was set to 100 %. Data are given as mean of three independent experiments ± SD. rANOVA: ** $p < 0,01$; *** $p < 0,001$

Figure S5: DMSO does not influence REDD1 regulation

HepG2 cells were treated for 30 min with DMSO (1 µl/ml) before stimulation with dexamethasone for 90 min and treatment with IL-6 for 75 min. REDD1 and HSC70 protein expression were evaluated by Western blotting. A representative result of $n = 3$ independent experiments is shown.

Figure S6: IL-6-induced reduction of REDD1 is independent of proteasomal degradation

For image quantification signals of REDD1, HSC70 as well as phosphorylated and total STAT3 as shown in Fig. 2B were analysed. **A)** shows the ratio of REDD1 to HSC70 and **B)** of phosphorylated STAT3 to STAT3. MG-115-induced expression of REDD1 as well as IL-6-induced phosphorylation of STAT3 was set to 100 %. Data are given as mean of three independent experiments ± SD. rANOVA: n.s.; ** $p < 0,01$; *** $p < 0,001$

Figure S7: Reduction of REDD1 expression is independent of caspase-mediated degradation

HepG2 cells were pretreated for 30 min with dexamethasone and subsequently stimulated with IL-6 for 75 min. REDD1 and HSC70 protein expression were evaluated by Western blotting. For detection of REDD1 a polyclonal antibody raised against full length REDD1 was used. A representative result of n = 3 independent experiments is shown.

Figure S8: The half-life of REDD1 is not influenced by IL-6

For image quantification signals of REDD1 and HSC70 as shown in Fig. 2C were analysed. The diagram shows the ratio of REDD1 to HSC70. Maximal expression of REDD1 was set to 100 %. Data are given as mean of three independent experiments ± SD. Student`s t test: n.s.

Figure S9: Half-life of exogenous REDD1

For image quantification signals of REDD1 and HSC70 as shown in Fig. 2D were analysed. The diagrams show the ratio of REDD1 to HSC70. Maximal expression of REDD1 was set to 100 %. Data are given as mean of three independent experiments ± SD.

Figure S10: IL-6 does not reduce expression of exogenous REDD1

For image quantification signals of REDD1, HSC70 as well as phosphorylated and total STAT3 as shown in Fig. 2E were analysed. **A)** shows the ratio of REDD1 to HSC70 and **B)** of phosphorylated STAT3 to STAT3. Expression of overexpressed REDD1 in the absence of IL-6 as well as maximal phosphorylation of STAT3 was set to 100 %. Data are given as mean of three independent experiments ± SD. rANOVA: n.s; *** $p < 0{,}001$

Figure S11: Knock-down of Dicer reduces miRNA processing

HepG2 cells were transfected with Ctr.-siRNA or Dicer-siRNA. 72 h after transfection cells were harvested. Dicer and HSC70 protein expression were evaluated by Western blotting. Total RNA containing miRNA was isolated using miRNeasy (Qiagen, Hilden, Germany) according to manufacturer`s instructions. mRNA was reverse transcribed into cDNA using miScript II RT KIT (Qiagen). Expression of mature miR18a_2 was analysed with a miScript Primer Assay (MS00031514) (Qiagen) and PCR was performed using miScript SYBR Green PCR Kit (Qiagen) on a Rotorgene (Qiagen). Expression of miR18a_2 was normalized against the mean CT values of five housekeeping genes using the ΔΔCT method. The following primers were used to amplify the cDNA of the housekeeping genes:

ACTB (fw: 5'-CTGGAACGGTGAAGGTGACA-3', rv: 5'-AAGGGACTTCCTGTAATAATGCA-3')
GAPDH (fw 5'-TGCACCACCAACTGCTTAGC-3', rv: 5'-GGCATGGACTGTGGTCATGAG-3')
HPRT (fw: 5'-TGACACTGGCAAAACAATGCA-3', rv: 5'-GGTCCTTTTCACCAGCAAGCT-3')
RPLI3A (fw: 5'-CCTGGAGGAGAAGAGGAAAGAGA-3', rv: 5'-TTGAGGACCTCTGTGTATTTGTCAA-3')
SDHA (fw: 5'-TGGGAACAAGAGGGCATCTG-3', rv: 5'-CCACTGTTGCTGCTGTCCAG-3').

Primers were obtained from MWG-Biotech (Ebersberg, Germany) and PCR was performed using Maxima SYBR Green (Thermo Fisher Scientific, Waltham, MA, USA) accoding to manufacturer`s

instructions. Maximal expression of mature miR18a was set to 100 %. Data are given as mean of two independent experiments ± SD. Student`s t-test: * $p < 0,1$.

Figure S12: REDD1 expression is increased in STAT3 deficient cells

MEF STAT3 $^{fl/fl}$, MEF STAT3$^{-/-}$ and MEF STAT3 $^{-/-}$ cells reconstituted with STAT3 were grown in DMEM (Thermo Fisher Scientific) supplemented with 10 % FCS, streptomycin (100 mg/ml) and penicillin (100 mg/ml) at 37°C in a water saturated atmosphere containing 10 % CO_2. Prior to stimulation cells were starved overnight in medium without FCS and phenol red. Subsequently cells were stimulated with IL-6 for 15 min. STAT3 and ERK1/2 phosphorylation as well as ERK1/2, HSC70, REDD1 and STAT3 protein expression were evaluated by Western blotting. A representative result of n = 3 independent experiments is shown.

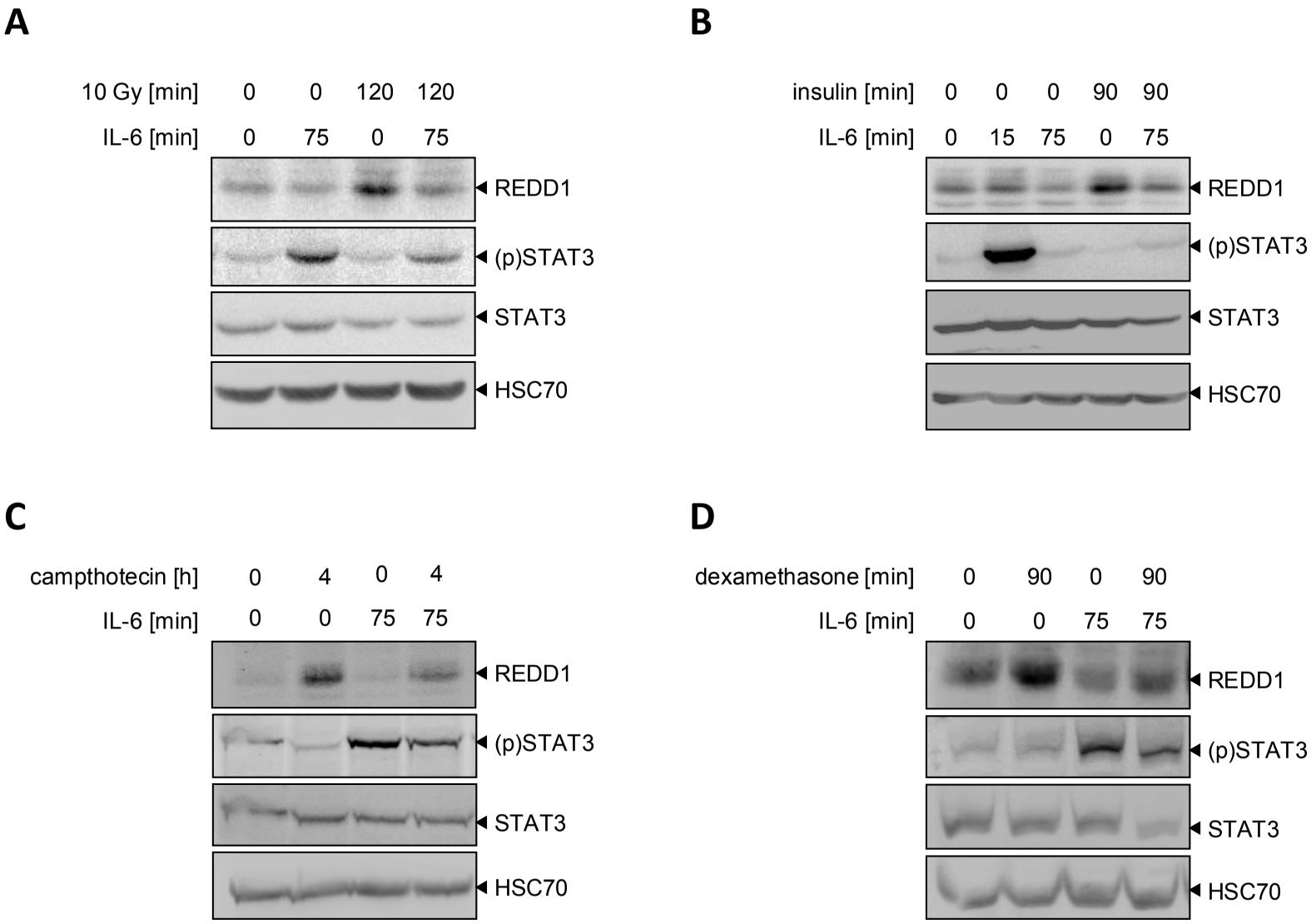

Fig. 1
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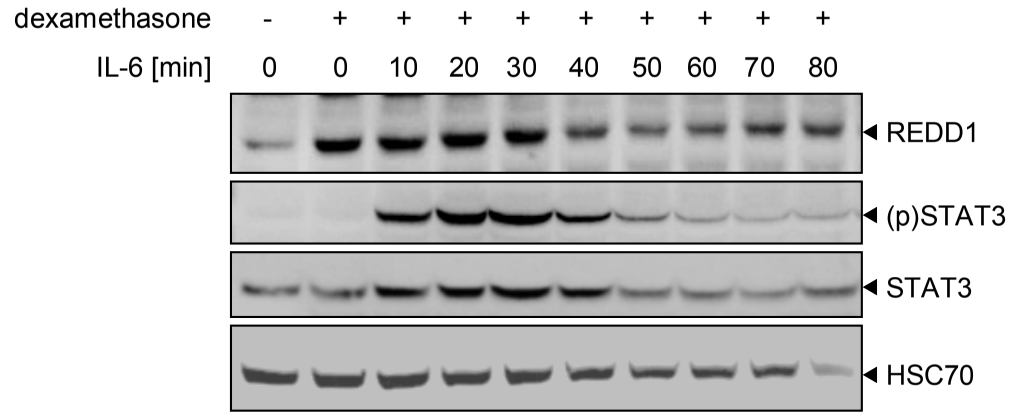
A



B

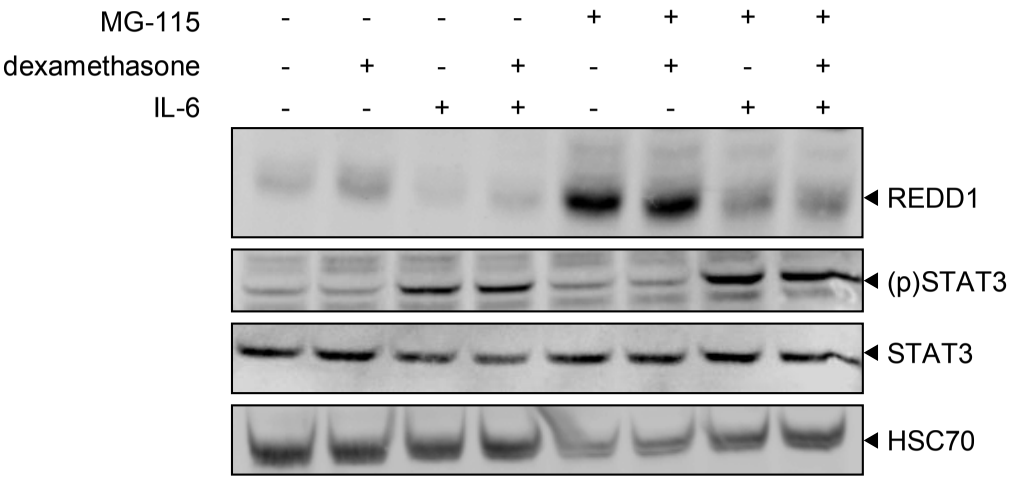


Fig. 2A,B
Pinno et al.

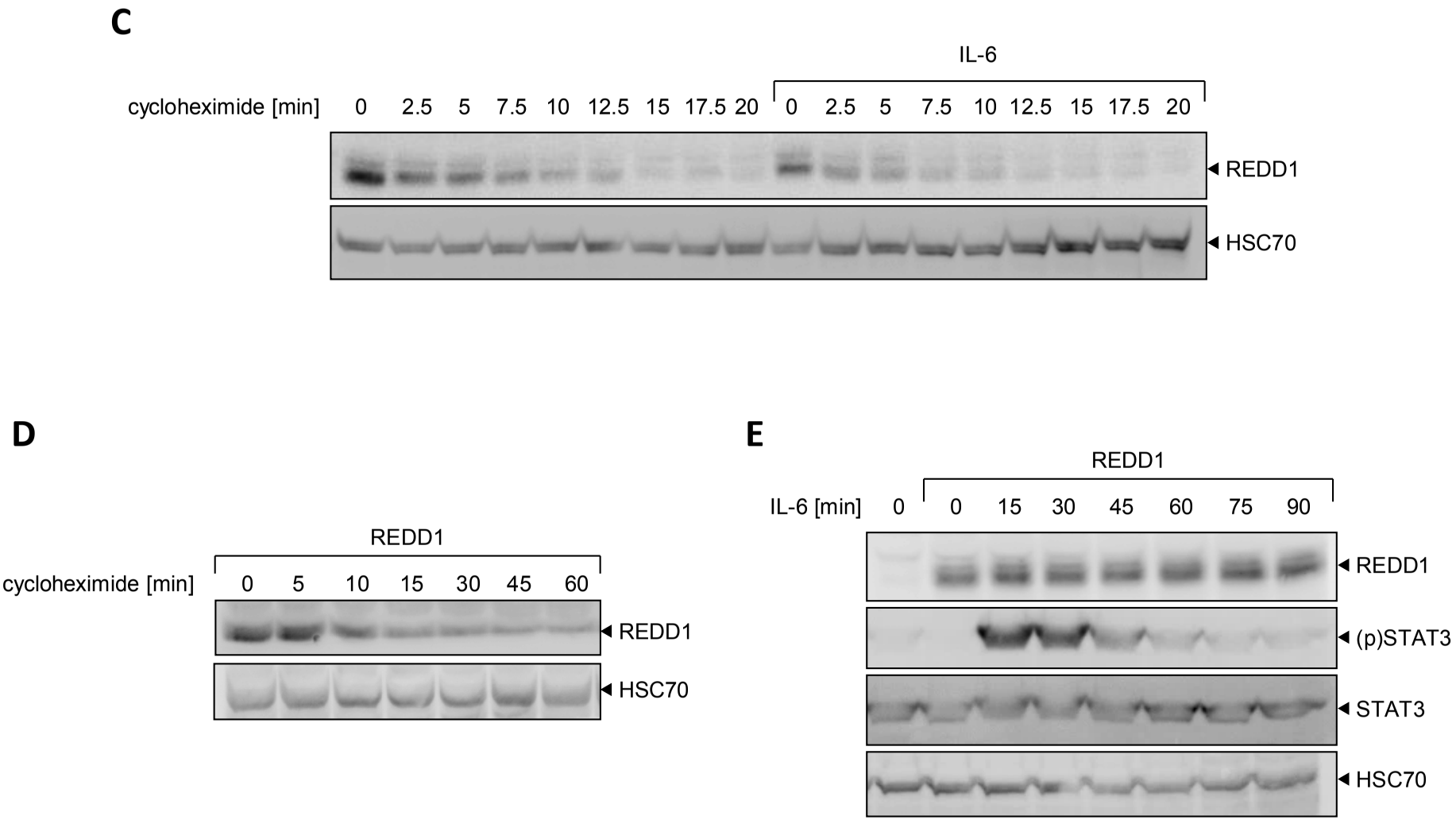


Fig. 2C,D,E
Pinno et al.

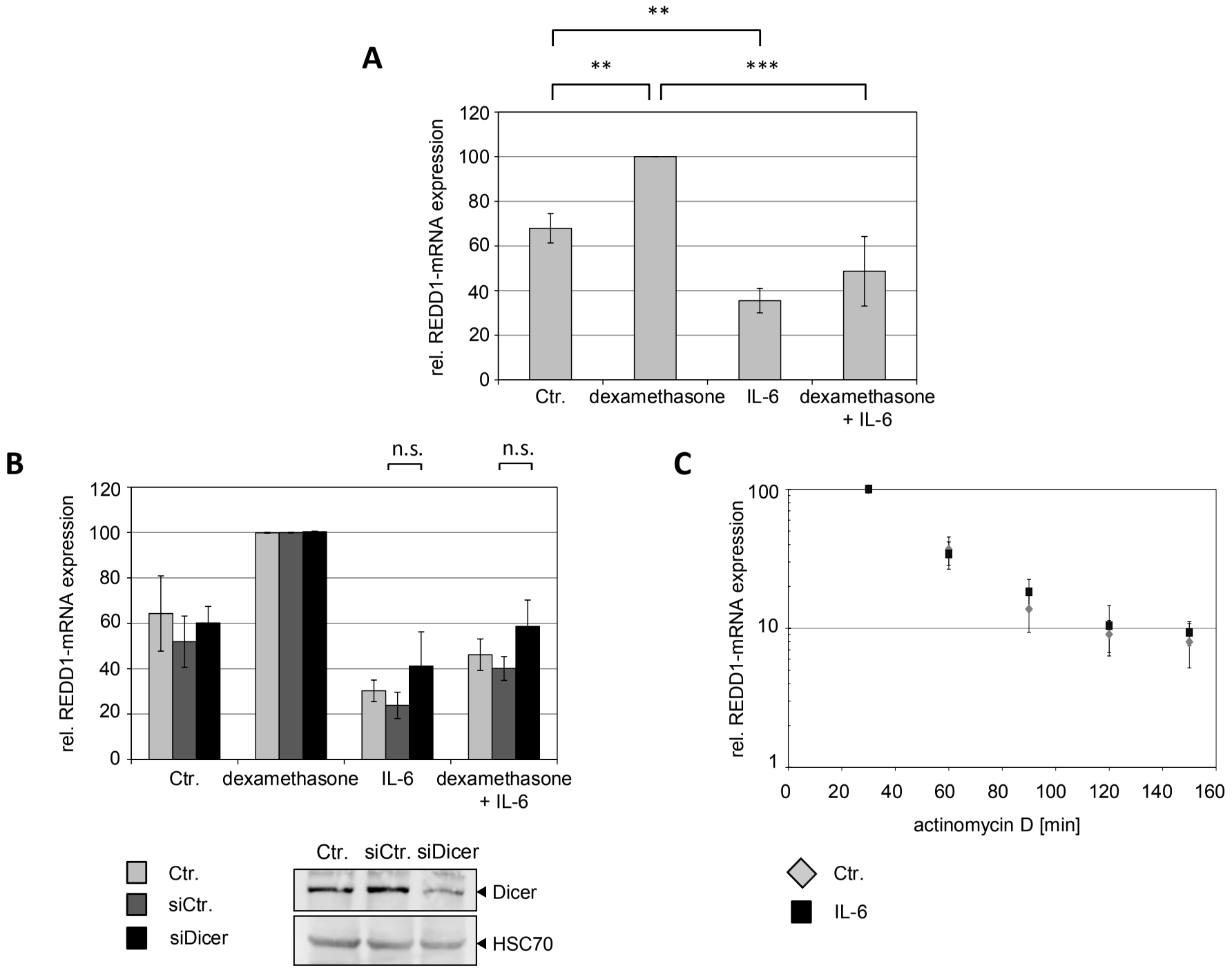


Fig. 3
Pinno et al.

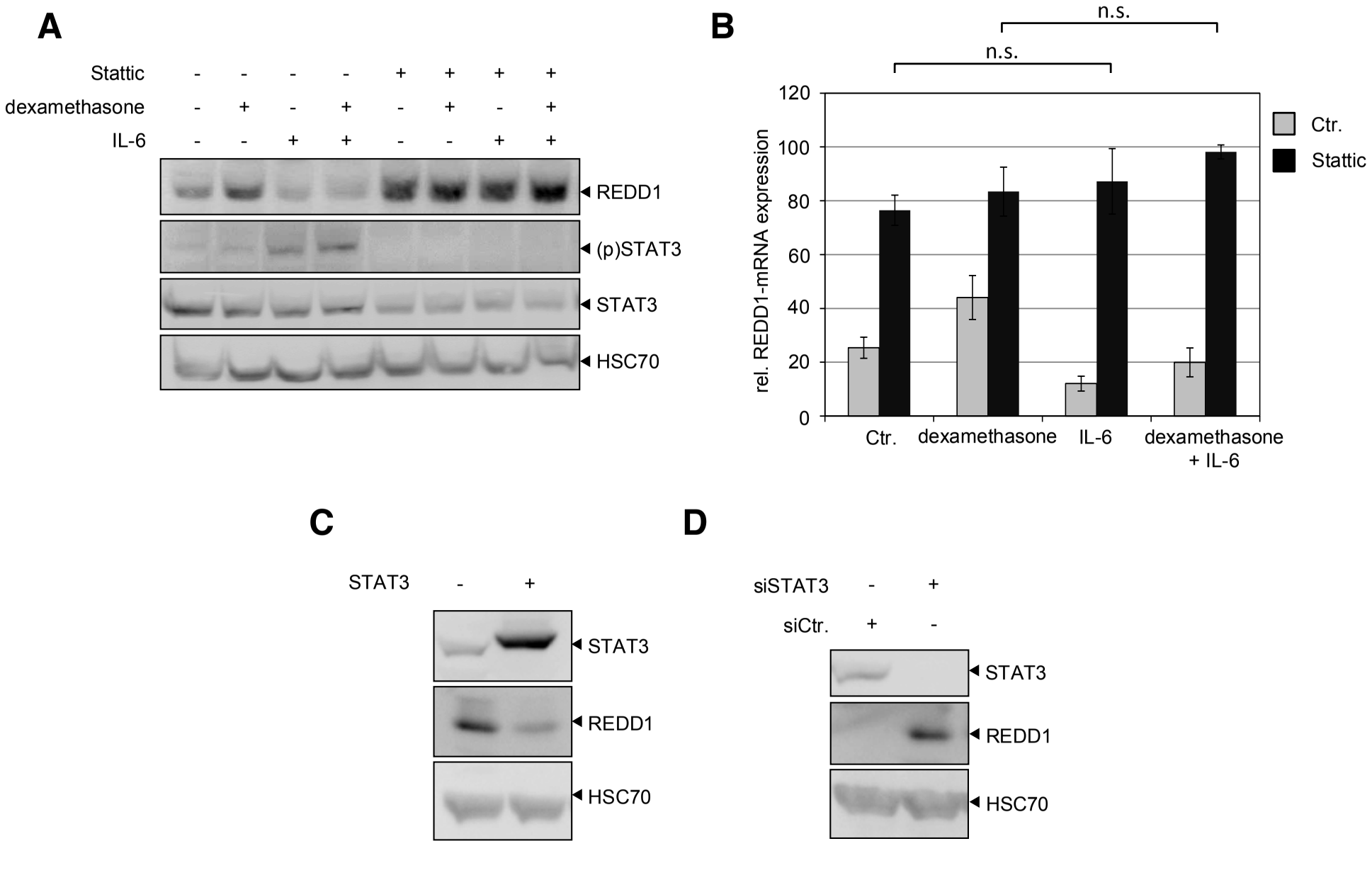


Fig. 4
Pinno et al.

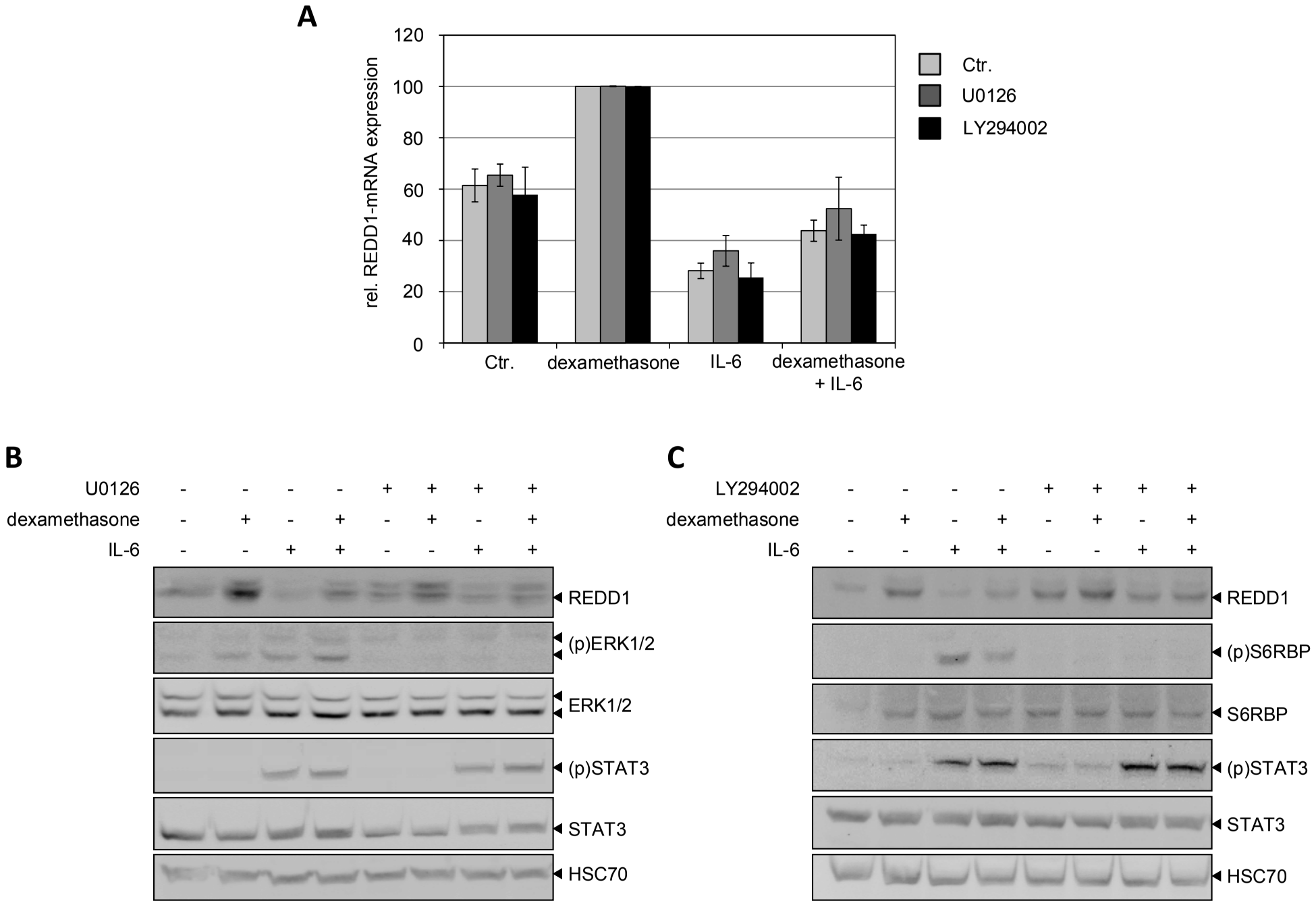


Fig. 5
Pinno et al.

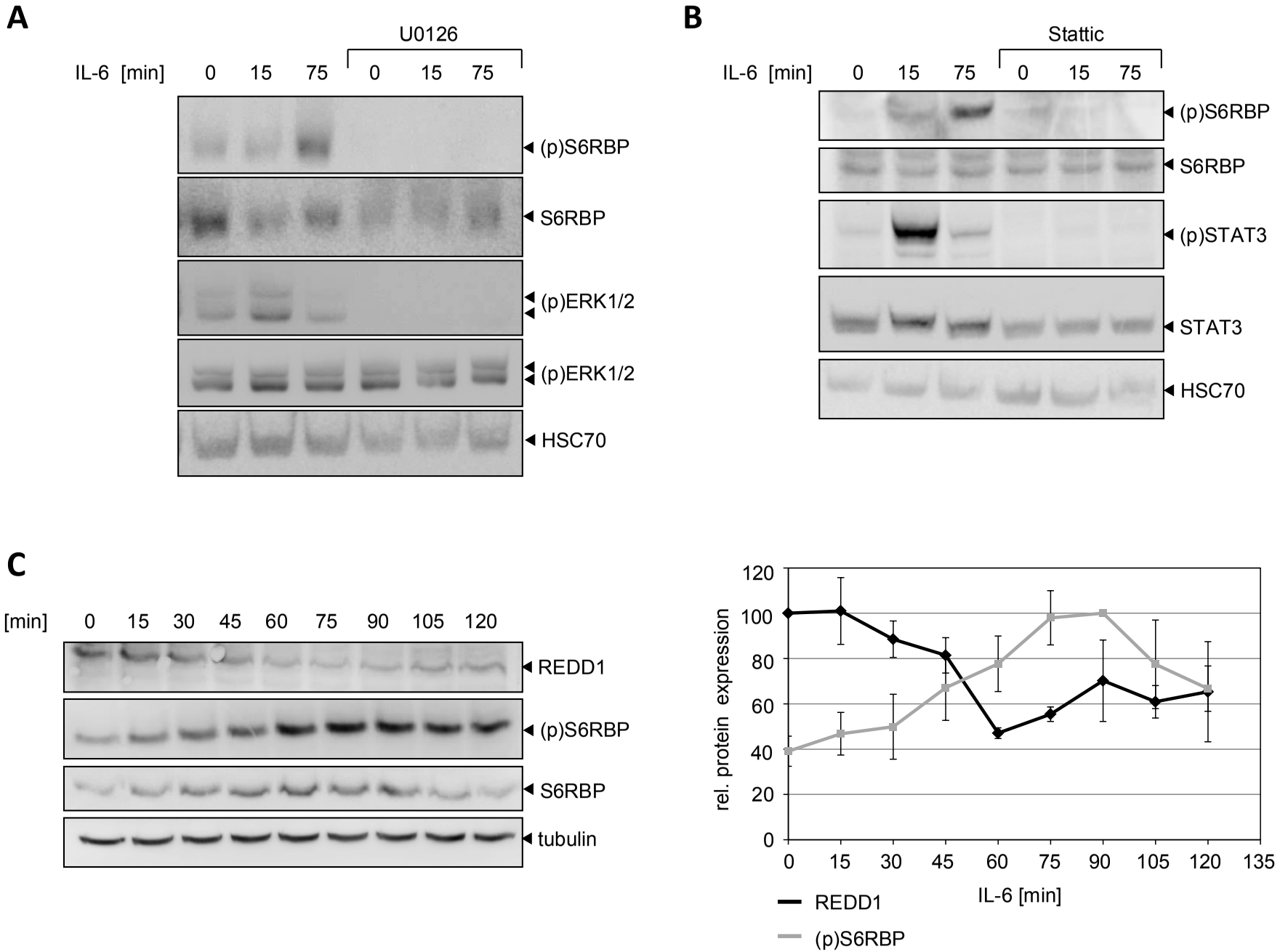


Fig. 6
Pinno et al.

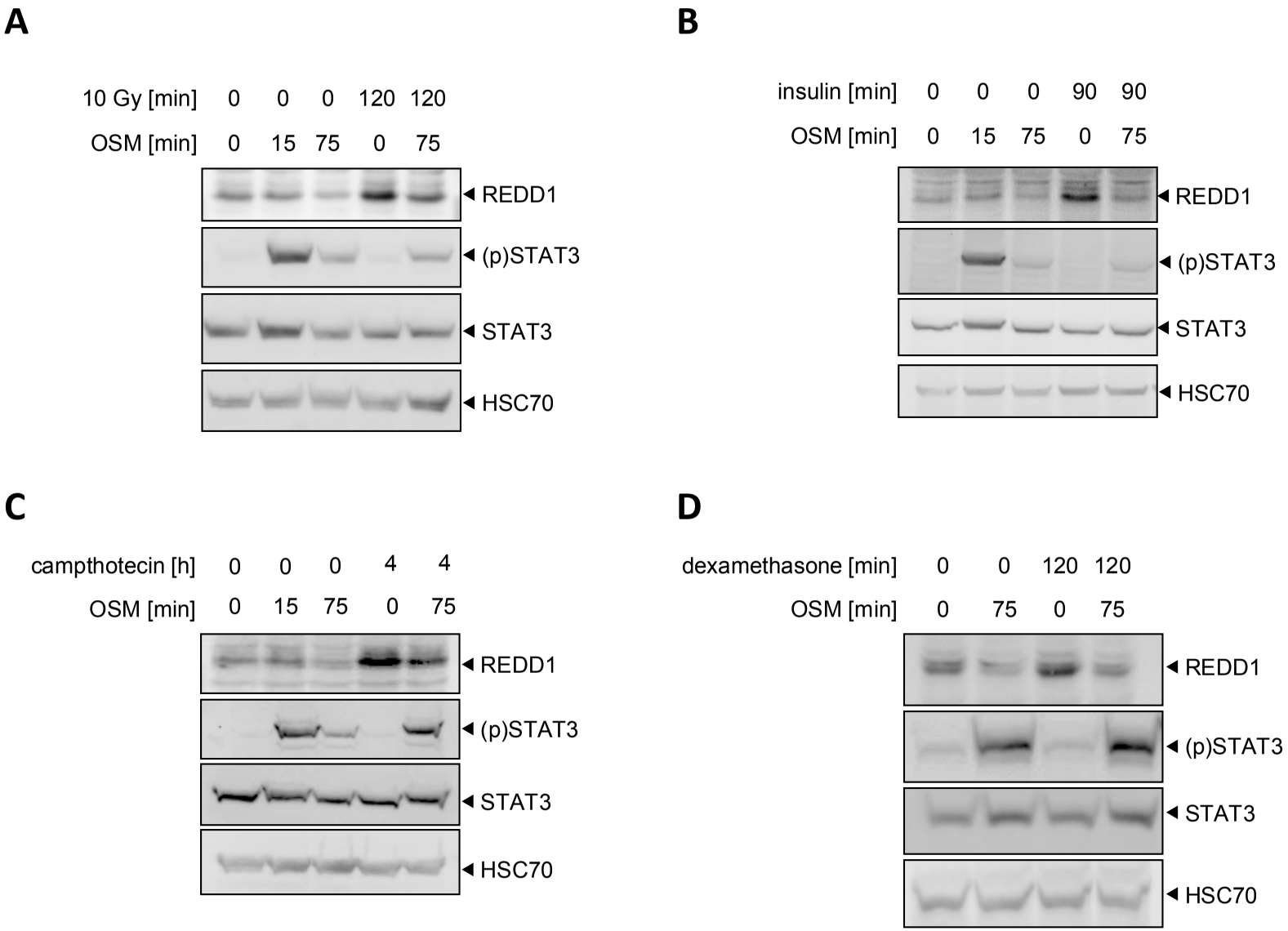


Fig. 7
Pinno et al.

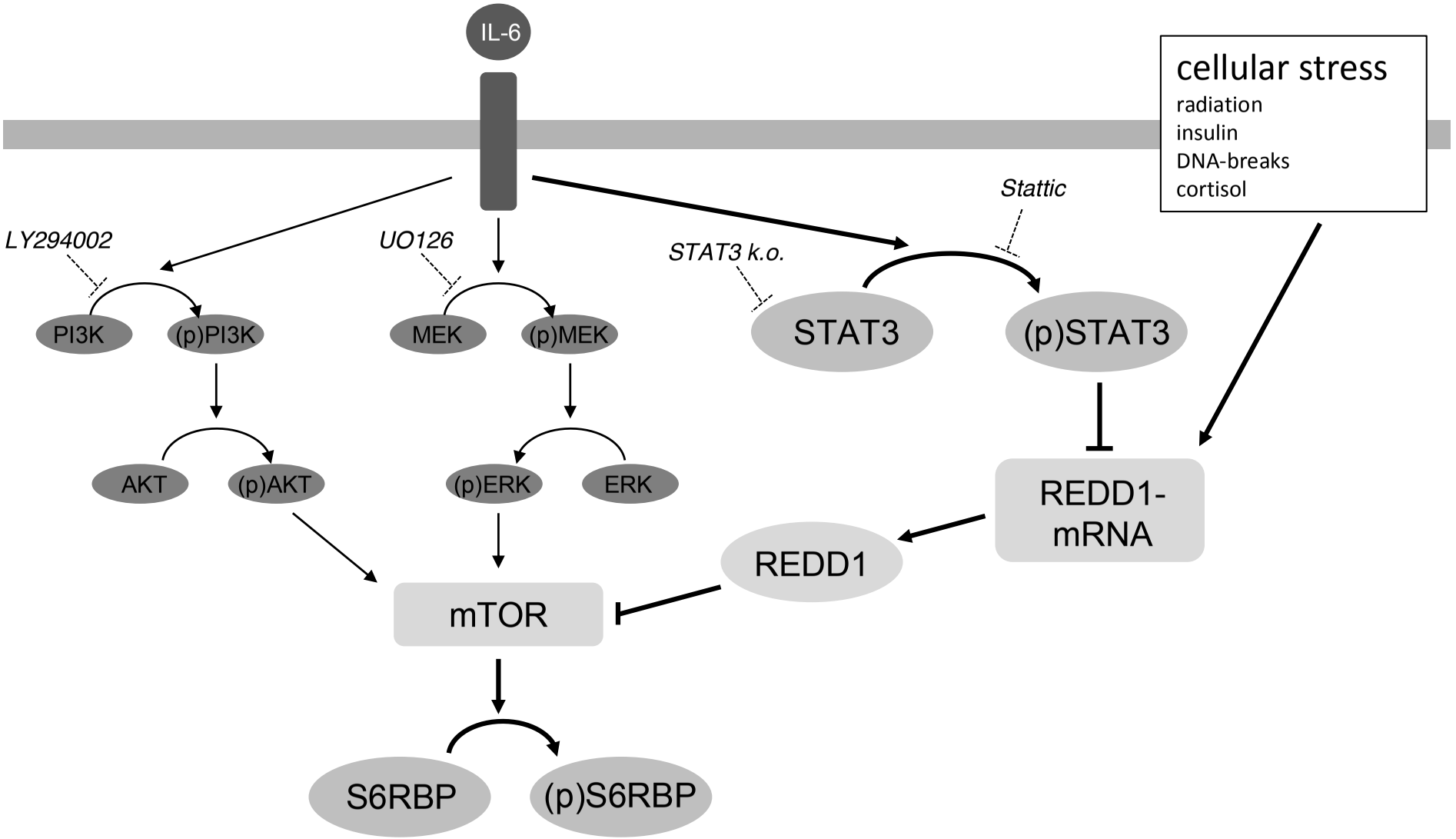


Fig. 8
Pinno et al.

Supl. Fig. 1

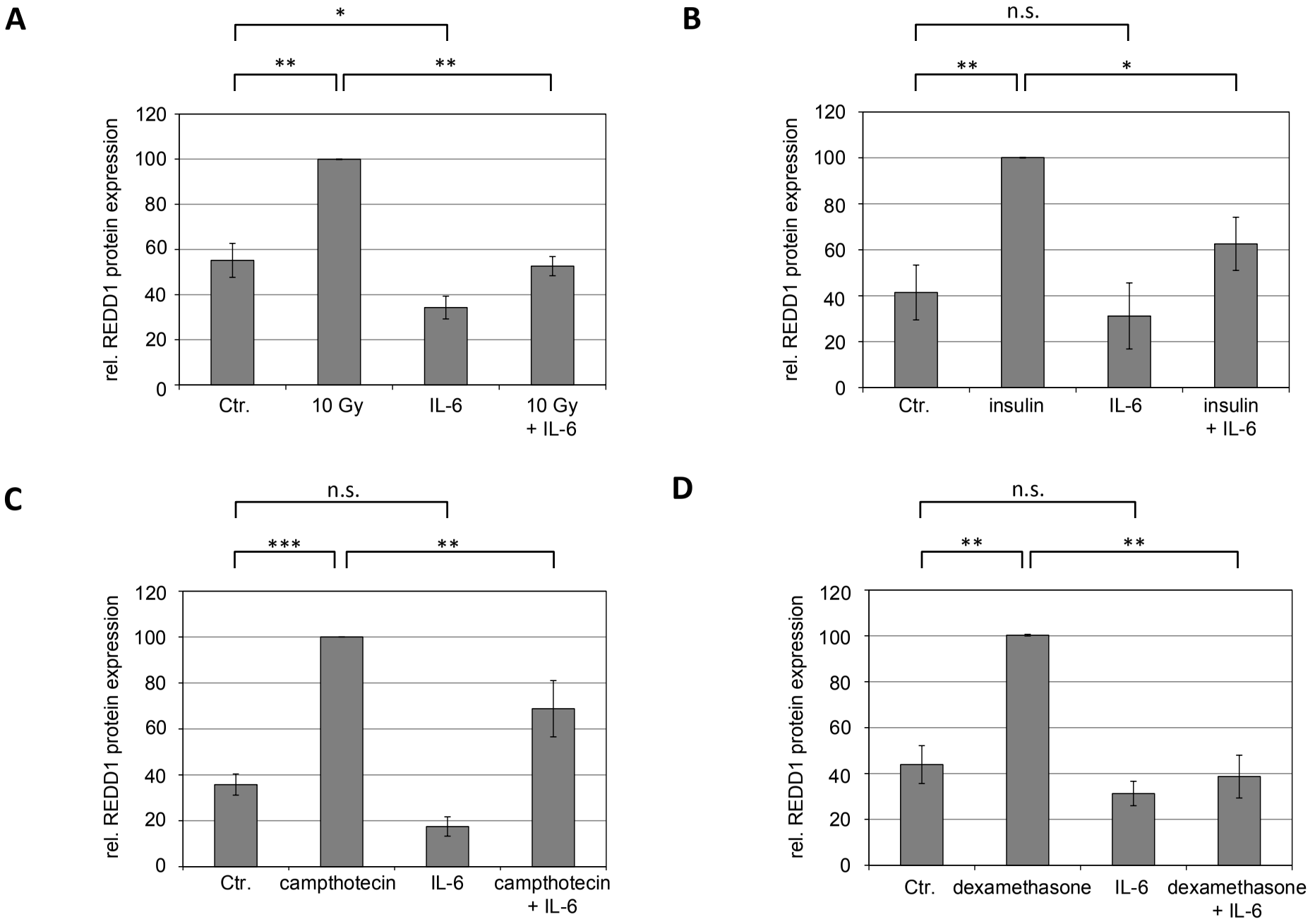


Fig. S1
Pinno et al.

Supl. Fig. 2

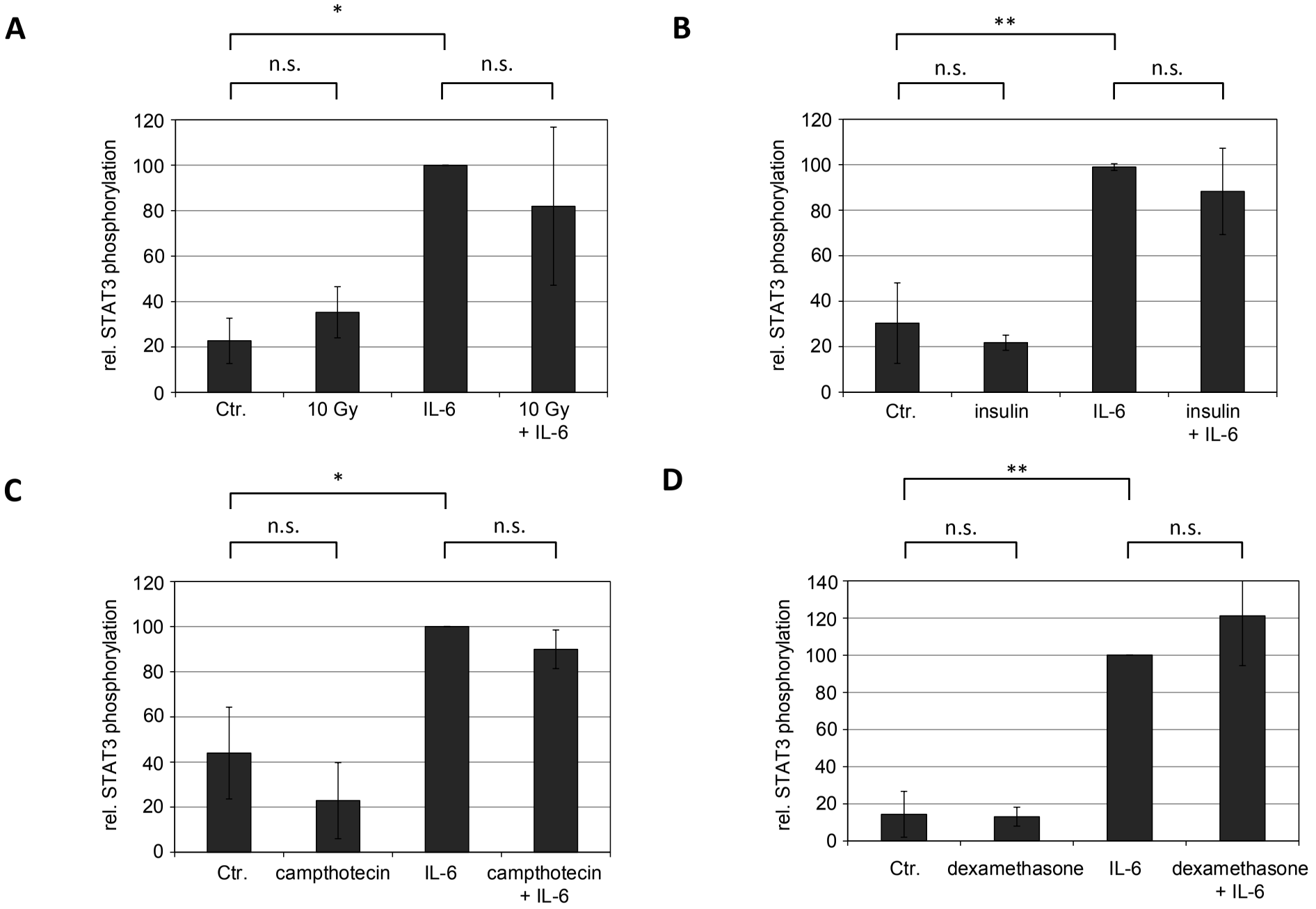
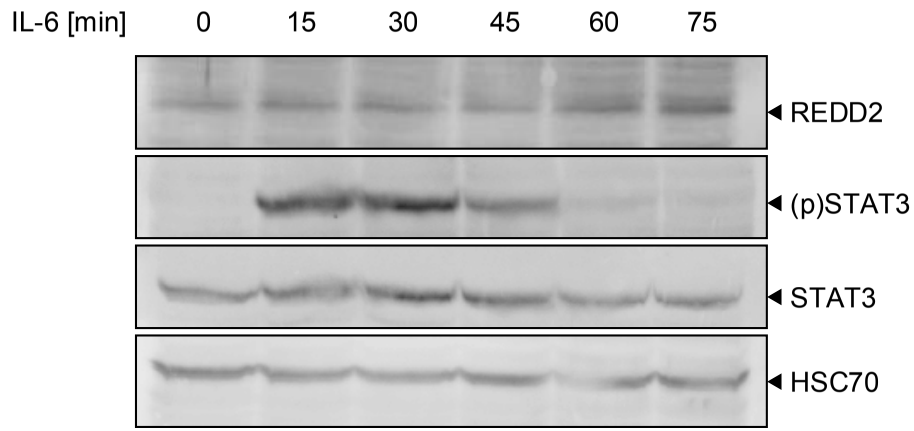


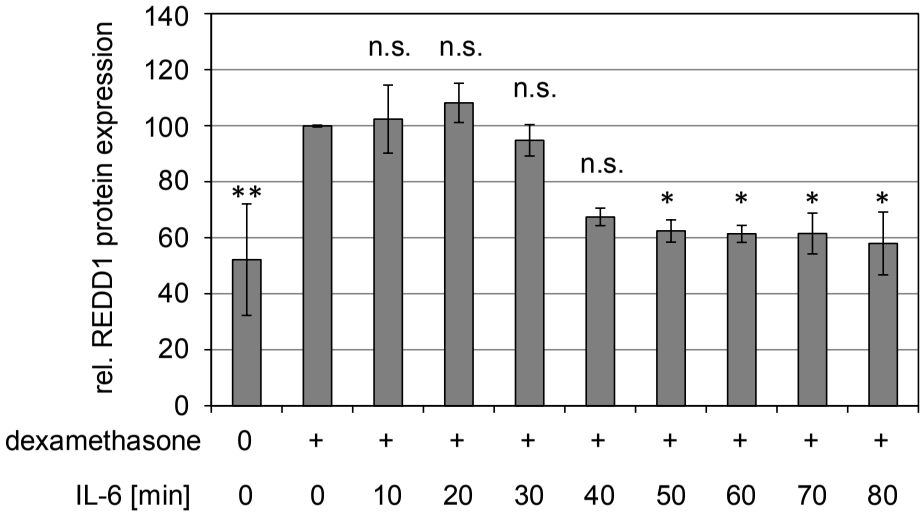
Fig. S2
Pinno et al.

Supl. Fig. 3



Supl. Fig. 4

A



B

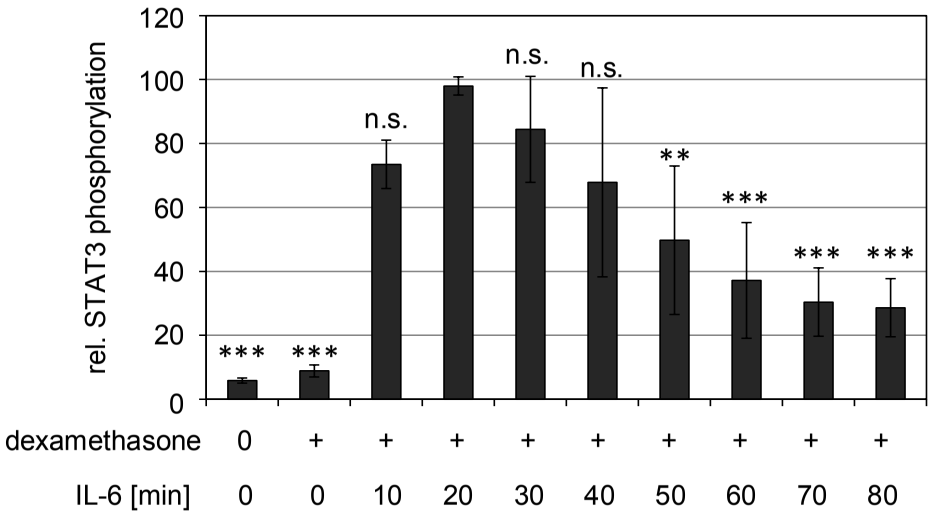
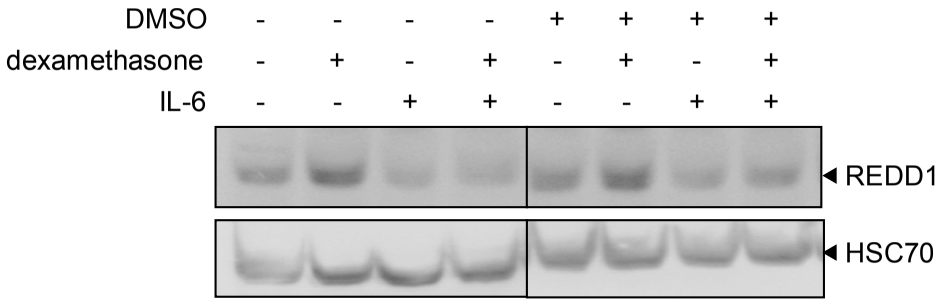


Fig. S3; Fig. S4
Pinno et al.

Supl. Fig. 5



Supl. Fig. 6

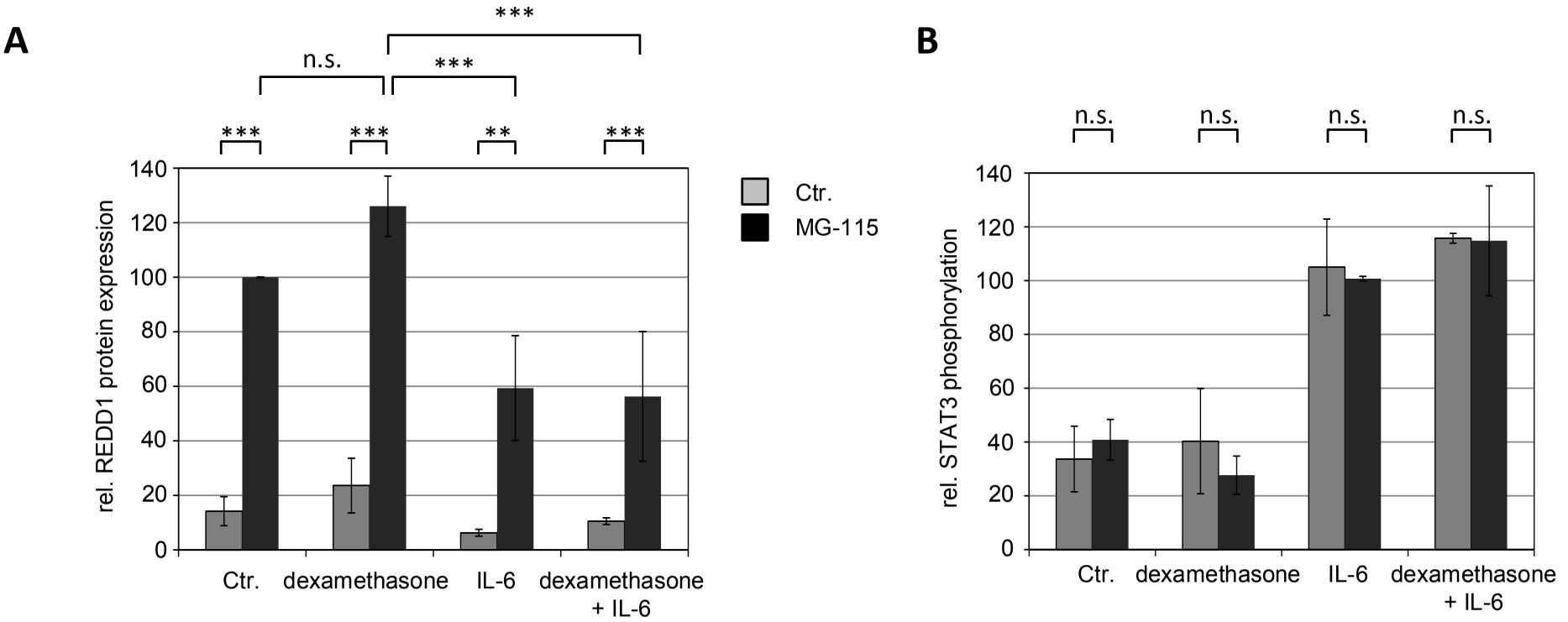
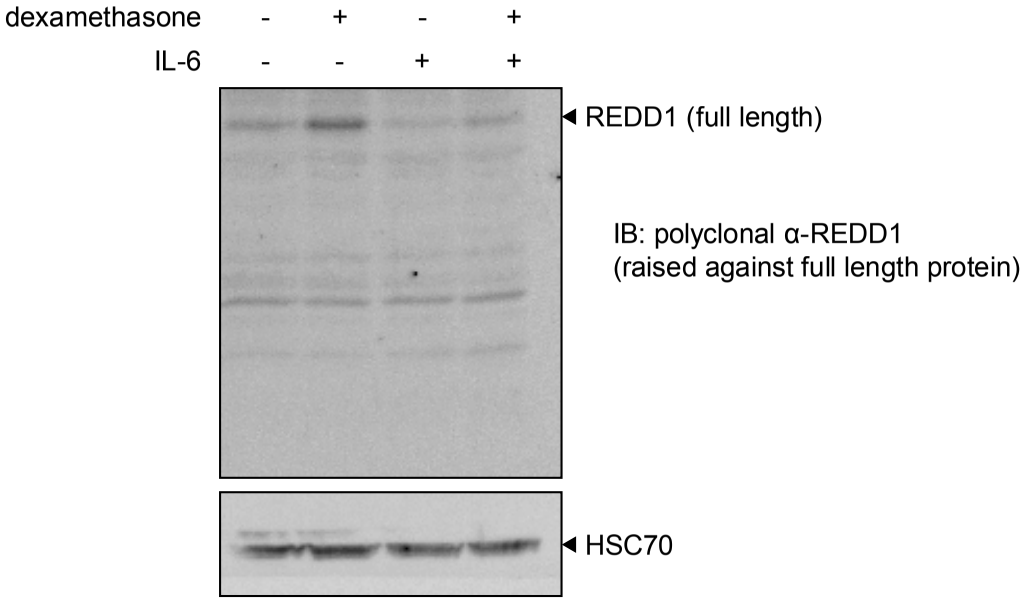
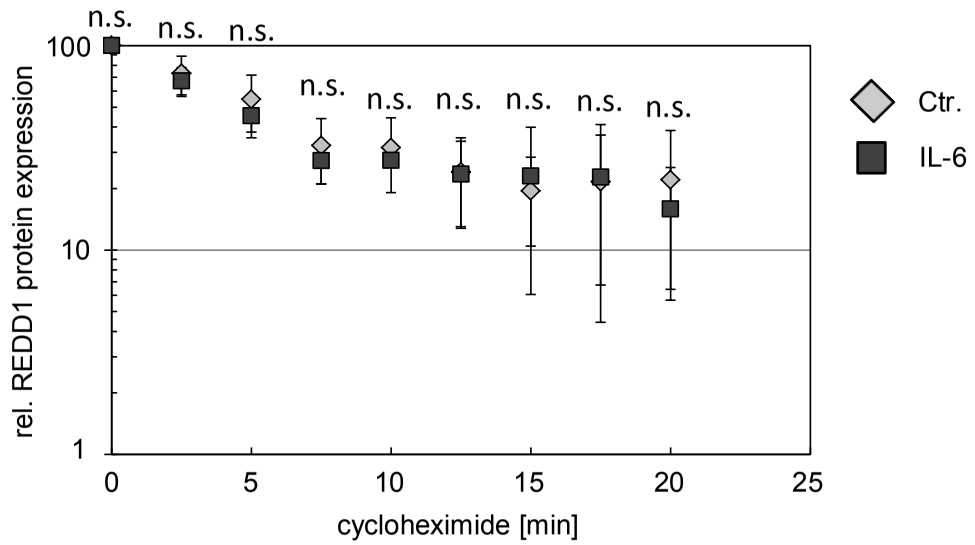


Fig. S5; Fig. S6
Pinno et al.

Supl. Fig. 7



Supl. Fig. 8



Supl. Fig. 9

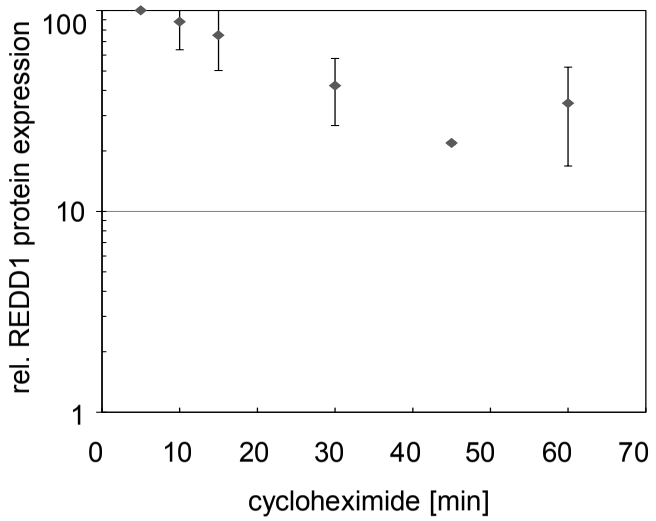
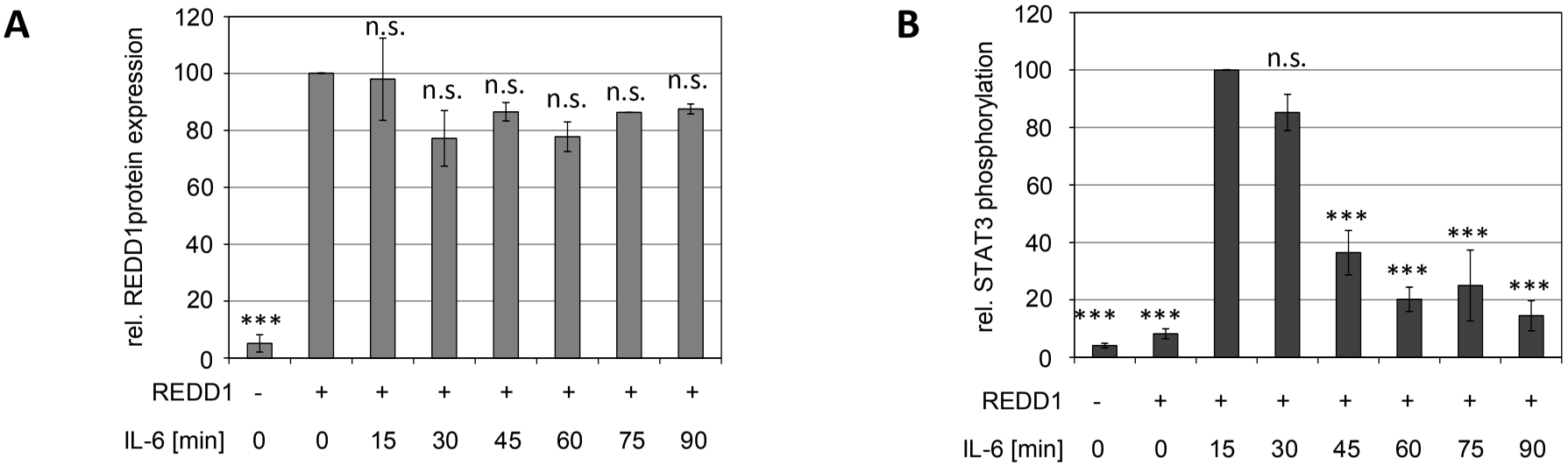
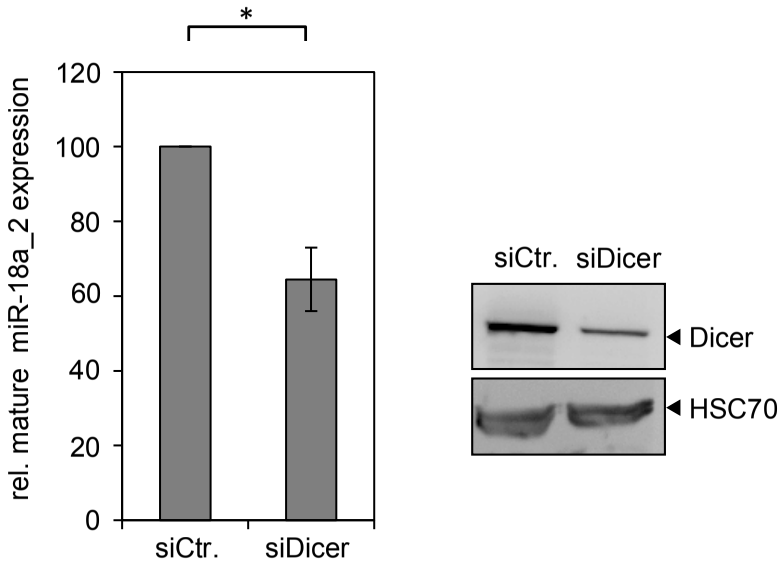


Fig. S7; Fig. S8; Fig. S9
Pinno et al.

Supl. Fig. 10



Supl. Fig. 11



Supl. Fig. 12

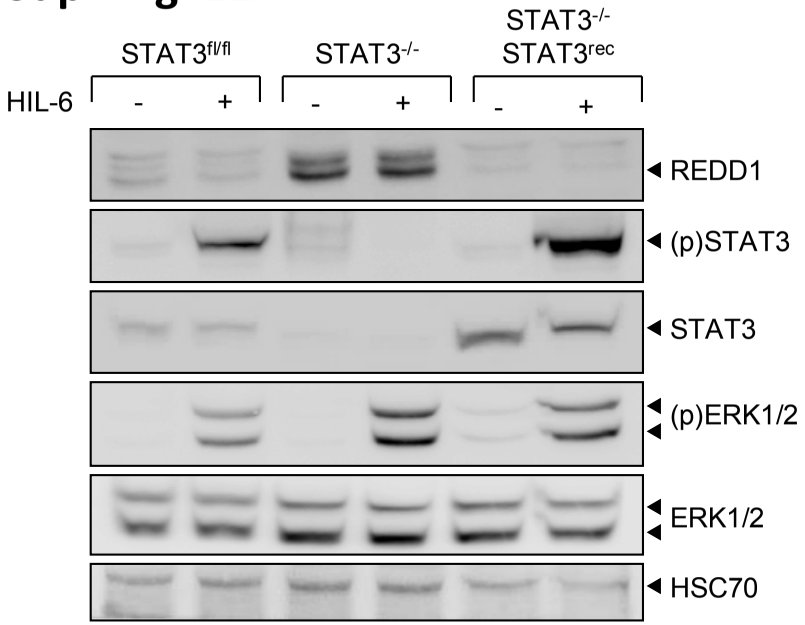


Fig. S10; Fig. S11; Fig. S12
Pinno et al.