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A new posttranslational regulation of REDD1/DDIT4 through cleavage by caspase 3 modifies its cellular function

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Dear Editor,

REDD1 (regulated in development and DNA damage responses 1; DDIT4) is a 232 amino-acid TSC2 activator and mTORC1-dependent signaling pathway inhibitor.[1,2] Although REDD1 expression has been characterized in response to hypoxia, DNA damage, and energy deprivation,[1,3,4] recent studies highlight new posttranslational mechanisms bearing on REDD1 stability.[5,6] To identify additional modifications contributing to REDD1 maintenance, we selected two human acute T-cell leukemia and chronic myeloid leukemia lines (JA3 and K562, respectively) expressing high endogenous REDD1 levels, and exposed both to pro- and anti-proliferative stimuli. Pro-apoptotic inducers Fas receptor activator (CH11) in JA3 cells (Figure 1a, left panel) and imatinib in K562 cells (Figure 1a, middle panel) decreased REDD1 protein levels and promoted accumulation of multiple lower bands detected around 17 kDa. V5-tagged REDD1-overexpressing HeLa cells treated with apoptosis-inducing ligand TRAIL clarified the accumulation of a triplet of bands at 17 kDa, identifiable as products of REDD1–V5 cleavage due to V5-directed antibody recognition (Figure 1a, right panel). Similar to procaspase 3 and caspase 3 substrate protein PARP, a positive control for apoptosis induction, REDD1 protein level remained unchanged in the presence of pan-caspase inhibitor zVAD-fmk, an effect observed in combination with each of the apoptosis-inducing agents tested, suggesting that caspases may directly cleave REDD1 during apoptotic activation.

To test this hypothesis, REDD1-encoding cDNA was transcribed/translated *in vitro* and the product of this reaction was incubated with recombinant caspases 3, 6, and 7. Only recombinant caspase 3 fully reproduced in a dose-dependent manner the pattern of REDD1 cleavage observed in intact cells, an effect blocked by zVAD-fmk (Figure 1b, upper left panel). We next mutated those REDD1 aspartate residues most conserved across species into non-cleavable alanines to identify sites directly targeted by caspase 3 (Figure 1b, lower left panel). Whereas REDD1 D58A, D74A, and D80A were partially resistant to caspase 3-mediated REDD1 cleavage, the triple mutant (TM, D58A/D74A/D80A) exhibited complete resistance, suggesting that caspase 3 cleaves REDD1 at each of these residues.

To assess the functional effects of this cleavage, we generated truncated forms of REDD1 corresponding to the cleavage products (Figure 1b, right panel). Only REDD1 ΔNter1 was stable following overexpression in cells; the others were readily degraded by the proteasome (not shown). Given the known function of REDD1 on mTOR pathway regulation, we overexpressed wild-type (WT), TM, and ΔNter1 REDD1 in HeLa cells exposed to brief amino-acid starvation or treated with insulin. As determined by phosphorylation levels of two mTOR downstream targets, p70S6K and RPS6, REDD1 WT and TM inhibited mTOR pathway, an effect enhanced upon insulin stimulation; REDD1 ΔNter1, however, lost the ability to modulate mTOR pathway activation (Figure 1c, left panel). Colony formation assays revealed that REDD1 ΔNter1 also conferred the highest anti-clonogenic potential (Figure 1c, right panel). Apart from known regulatory effects on the mTOR pathway, REDD1 cleavage by caspase 3 therefore unmasks a new role for REDD1 in cell survival alteration. Recent findings suggest that REDD1 may interact directly with mitochondrial metabolism, as endogenous REDD1 can localize to mitochondria.[7] We found that overexpression of REDD1 ΔNter1 rendered HeLa cells more sensitive to low doses of TRAIL and staurosporine (not shown), suggesting that cleaved REDD1 ΔNter1 may alter mitochondrial pathways to promote drastic anti-proliferative properties and, within the context of apoptosis induction, act as

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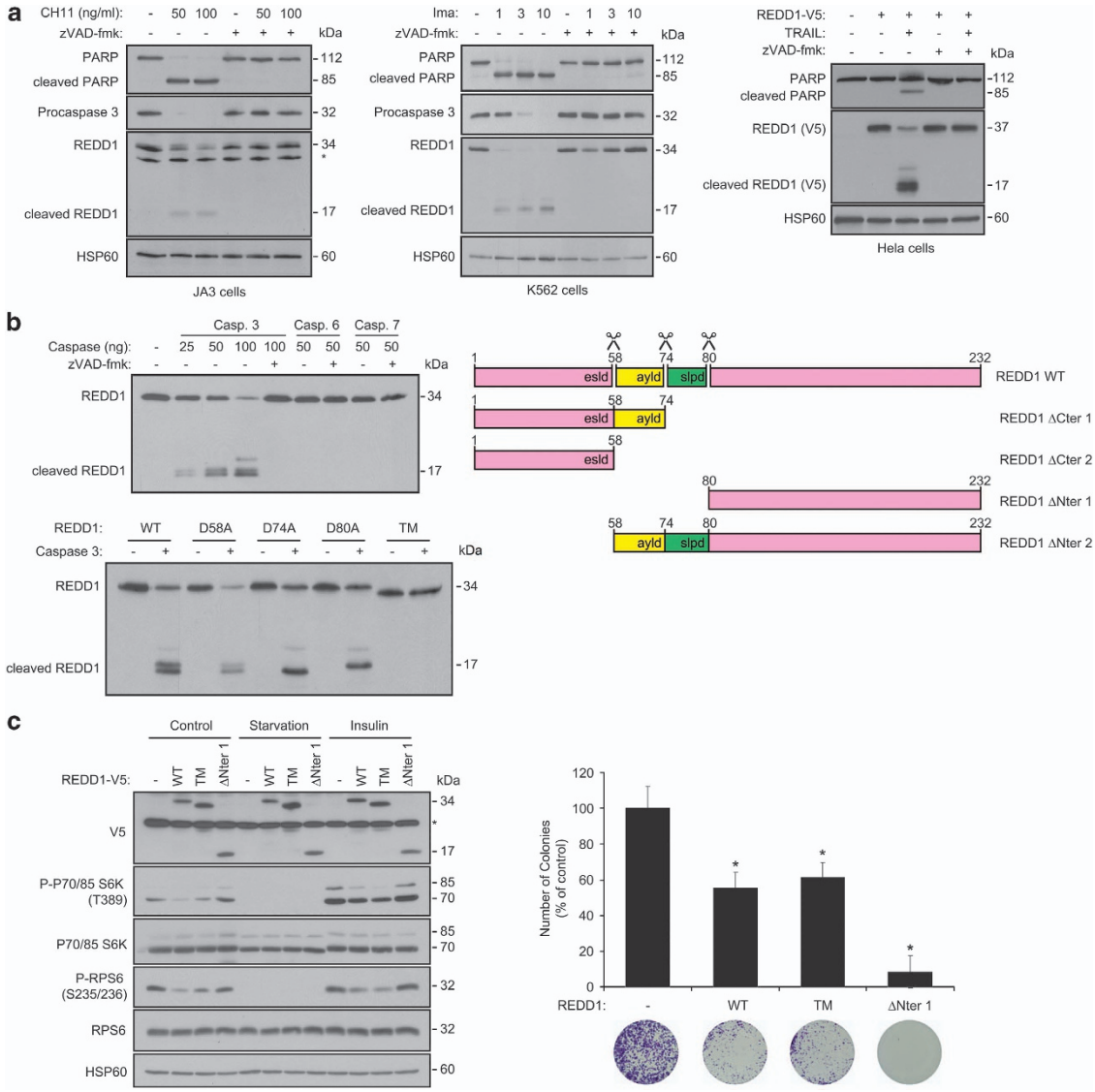

Figure 1 (**a**, left panel) Western blot for indicated proteins on lysates from JA3 cells co-treated for 8 h with various concentrations of anti-human Fas-activating antibody, CH11 (Millipore, Billerica, MA, USA), and 50 μM pan-caspase inhibitor, zVAD-fmk (Enzo Life Sciences, Inc., Farmingdale, NY, USA). *, nonspecific band. (**a**, middle panel) Western blot for indicated proteins on lysates from K562 cells co-treated for 24 h with various concentrations of Imatinib (Novartis Pharma, Basel, Switzerland) and 50 μM zVAD-fmk. (**a**, right panel) Western blot for indicated proteins on lysates from REDD1-V5-overexpressing HeLa cells co-treated for 8 h with 50 ng/ml TRAIL (TNF-related apoptosis-inducing ligand) and 50 μM zVAD-fmk. (**b**, upper left panel) Western blot for REDD1 transcribed and translated *in vitro* with ^{35}S-methionine and then incubated for 6 h at 37 °C in 50 μl of 25 mM HEPES pH7.5, 0.1% CHAPS, 5 mM DTT with indicated recombinant caspases in the presence or absence of 50 μM zVAD-fmk. (**b**, lower left panel) Western blot for REDD1 WT or four REDD1 D58A, D74A, D80A, and TM (triple mutant, D58A/D74A/D80A) mutants transcribed and translated *in vitro* with ^{35}S-methionine and incubated with indicated recombinant caspases for 6 h at 37 °C. (**b**, right panel) Schema depicting the location of the three caspase 3-targeted aspartate residues on REDD1 protein and the structure of the four major caspase 3-produced fragments that were subcloned for further investigation in this study. (**c**, left panel) Western blot for downstream mTOR pathway targets (p70S6K and RPS6) on lysates from HeLa cells overexpressing REDD1 WT, TM, and ΔNter1 constructs either control, amino acids starved for 6 h or treated with 100 ng/ml insulin for 15 min post serum starvation. *, nonspecific band. (**c**, right panel) HeLa cells were co-transfected with a combination of pBABE-puro and indicated REDD1 constructs (ratio 1 : 10). After selection with puromycin, colonies were scored by ImageJ quantification software (US National Institutes of Health, Bethesda, MD, USA). *$P < 0.05$ calculated using a Mann–Whitney test

a pro-apoptotic amplification signal. Moreover, *in* and *ex vivo* studies show that *Redd1* knockout cells undergo a more pronounced apoptosis than *Redd1* WT cells in response to dexamethasone or doxorubicin,[3,8] confirming that anti-apoptotic function is dampened by caspase 3-mediated destabilization. The REDD1 ΔNter1 product not only marks the loss of REDD1 anti-apoptotic function, but may also act as a trigger that itself initiates robust deleterious effects.

Conflict of Interest

The authors declare no conflict of interest.

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