

Caspase-2 is required for DNA damage-induced expression of the CDK inhibitor p21^{WAF1/CIP1}

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ABSTRACT

Although caspase-2 represents the most conserved caspase across species and was the second caspase identified, its precise function remains enigmatic. In several cell types we show that knockdown of caspase-2 specifically impaired DNA damage-induced p21 expression, while overexpression of a caspase-2 mutant increased p21 levels. Caspase-2 did not influence p21 mRNA transcription, and also various inhibitors targeting proteasomal or non-proteasomal proteases including caspases could not restore p21 protein levels following knockdown of caspase-2. As, however, silencing of caspase-2 only impaired exogenous expression of p21 constructs containing 3'-UTR sequences, our results strongly indicate that caspase-2 regulates p21 expression at the translational level. Intriguingly, unlike depletion of caspase-2 that prevented p21 expression and thereby reverted the γ -irradiation-induced senescent phenotype of wild-type HCT116 colon carcinoma cells into apoptosis, neither knockdown of the caspase-2-interacting components RAIDD, RIP or DNA-PKcs was able to mimic these processes. Together, our data suggest that this novel role of caspase-2 as a translational regulator of p21 expression occurs not only independently of its enzymatic activity, but does also not require known caspase-2-activating platforms.

INTRODUCTION

Although caspases were initially identified as key apoptotic enzymes, they are now known to also exert essential functions in diverse cellular processes including inflammation, differentiation and proliferation.¹ Among them, caspase-2 is not only the most conserved caspase across species, but intriguingly also the most enigmatic, as it combines features of both initiator and executioner caspases.^{2,3} It contains a long caspase activation and recruitment domain (CARD), a characteristic trait of initiator caspases required for their activation, while its cleavage specificity resembles that of effector caspases. Similar to initiator caspases such as caspase-8, -9 and -10, caspase-2 is activated by dimerization via a so-called “induced-proximity”-mechanism, although cleavage of caspase-2 was found to stabilize the active protease.^{4,5} In contrast, as caspase-2 is a poor activator of effector caspases, it surely lacks an essential feature of initiator caspases. Thus, not unexpectedly, contradictory findings have been reported that place caspase-2 either upstream or downstream of mitochondria, or even fail to assign any specific function to this enzyme in apoptosis.^{6,7}

In sharp contrast to the *in utero* or perinatal lethality of *Casp8*⁸ and *Casp9*-knockout mice,^{9,10} *Casp2*-knockout mice were found to be viable and develop normally, and thymocytes from these animals show no obvious apoptosis defects compared to their wild-type littermates.^{11,12} Female *Casp2*-deficient animals, however, display a slight increase in their oocyte numbers probably due to an impaired apoptosis program,¹¹ and mice lacking caspase-2 show signs of premature ageing.¹³ Also other *Casp2*-deficient cell types such as neurons or embryonic fibroblasts (MEFs) show a reduced or delayed apoptotic response toward certain stimuli,^{6,7} indicating both redundant and non-redundant functions for caspase-2 in apoptosis. In addition caspase-2 was reported to be involved in the regulation of DNA damage responses and cell cycle progression and may even act as tumor suppressor.^{14,15} However, the underlying mechanisms are completely unresolved mainly due to the fact that only few caspase-2-specific substrates have yet been identified.

Unlike caspase-8 and -9 that become activated in the death-inducing signaling complex (DISC) and the apoptosome, activation of caspase-2 is achieved via formation of

three functionally different multi-protein complexes. The first caspase-2-activating complex identified was the so-called RAIDD-PIDDosome consisting of RAIDD [receptor-interacting protein (RIP)-associated ICH/CED-3-homologous protein with death domain], PIDD (p53-induced protein with death domain), and caspase-2.¹⁶ The RAIDD-PIDDosome is formed in response to genotoxic stressors and induces apoptosis most likely via caspase-2-mediated BID cleavage.¹⁷ However, caspase-2 was also found processed and capable of inducing apoptosis in the absence of RAIDD or PIDD, suggesting the existence of additional caspase-2-activating platforms.¹⁸ Thereby, caspase-2 was proposed to form a cytosolic complex with RIP and TRAF-2 (TNF receptor-associated factor-2) that, however, does not induce apoptosis, as it strongly activates NF- κ B and p38.¹⁹ Surprisingly, this was independent of the proteolytic activity of caspase-2, and instead required only the prodomain containing the CARD oligomerization motif.

In contrast to these cytosolic complexes, a third caspase-2-activating complex formed in the nucleus was implicated in the maintenance of a G2/M DNA damage checkpoint and in DNA repair executed by the NHEJ pathway.¹⁴ Thereby, the DNA-dependent serine/threonine protein kinase (DNA-PKcs) and PIDD form the backbone of the so-called DNA-PKcs-PIDDosome that is constitutively present even in unstimulated cells. Following DNA damage, caspase-2 is recruited and activated in this complex via the DNA-PKcs-mediated phosphorylation of its Ser122 residue. Even though the identification of the DNA-PKcs-PIDDosome resolved another enigma surrounding caspase-2, namely its constitutive nuclear localization, it remains unanswered of how nuclear caspase-2 is integrated in the DNA damage-induced G2/M checkpoint and NHEJ pathways.

Although originally identified as a cyclin-dependent kinase (CDK) inhibitor mediating p53-induced cell cycle arrest, p21 is now known to also participate in diverse biological processes including transcription, DNA repair, differentiation, senescence and apoptosis.²⁰⁻²² Thus, mechanisms are required that tightly control expression and functional diversity of p21. This is achieved for instance by multiple phosphorylations that either result in its stabilization or an increased degradation by the proteasome.²³ In addition to this post-

translational regulation, expression of p21 is also controlled at the transcriptional and post-transcriptional level,^{24,25} giving the cell multiple opportunities to interfere with p21 function.

Here we uncovered an unprecedented role of caspase-2 as an essential translational cofactor for DNA damage-induced p21 expression. This novel role that enables caspase-2 to control the diverse activities of p21 and that does not depend on its activation in known caspase-2-activating platforms was observed in different human cell types independently of the kind of DNA damage applied. Thus, our findings add a new level of complexity to the enigma surrounding the role(s) of caspase-2 and establish a novel apoptosis-independent involvement of this protease in cellular DNA damage signaling pathways.

RESULTS

Caspase-2 knockdown sensitizes wild-type HCT116 cells to γ IR-induced apoptosis by reducing p21 protein levels.

Recently we reported that besides the induction of p53-dependent senescence, p21 is also required for the simultaneous inhibition of caspase activation and apoptosis downstream of mitochondria.²⁶ This conclusion was drawn from our finding that only checkpoint-deficient HCT116 cells (p21^{-/-} and p53^{-/-}) succumb to apoptosis upon exposure to ionizing radiation (γ IR), whereas similarly treated wild-type cells are driven into senescence. To verify that the hypersensitivity of p21-deficient HCT116 cells is indeed due to the loss of p21 and not caused primarily by the elevated levels of p53 and Puma (Fig. 1A),²⁶ we silenced p21 expression in wild-type cells by RNA interference. Knockdown of p21, but not a control siRNA reverted the senescent phenotype of irradiated wild-type HCT116 cells and sensitized them to apoptosis as evidenced by the processing and activation of caspase-9 and -3 (Fig. 1B,C).

More interestingly, following γ IR exposure procaspase-2 levels decreased in a time-dependent manner in both HCT116 lines regardless of whether or not they succumbed to apoptosis (Fig. 1A,B), implying processing of caspase-2 (please see discussion for further details). As in contrast, procaspase-9 and -3 are processed and activated exclusively in apoptotic checkpoint-deficient cells, and because γ IR induces activation of mitochondria even in HCT116 wild-type cells that become senescent upon this treatment,²⁶ our data imply an important upstream role for caspase-2 in DNA damage signaling pathways.

To more closely examine this role, we compared the fate of irradiated HCT116 wild-type cells in the presence and absence of caspase-2. Surprisingly, siRNA-mediated silencing of caspase-2 reverted the senescent phenotype of irradiated wild-type cells and resulted in the processing and activation of caspase-3 as well as in the death-associated release of the lactate-dehydrogenase (LDH) (Fig. 2A-C). This phenomenon could not be explained by an elevated

expression of p53 or Puma, because their γ IR-induced protein levels did not differ significantly in the absence or presence of caspase-2 (Fig. 2A). In contrast, wild-type cells sensitized to γ IR-induced apoptosis by knockdown of caspase-2 displayed a remarkable strong decrease in p21 protein expression (Fig. 2A). This was also observed when individual siRNAs were used that comprise the caspase-2 SMARTpool siRNA ruling out off-target effects (Suppl. Fig. 1). On the other hand, p21 levels increased following overexpression of caspase-2, however, only in unstressed cells and when a catalytically inactive caspase-2 mutant was used (Fig. 2D). This most likely reflects the facts that irradiation per se induces the maximum level of p21 expression in HCT116 wild-type cells that cannot be further enhanced by exogenous caspase-2 and that cells transfected with the wild-type enzyme undergo massive apoptosis (not shown) explaining not only its weak detection, but also the minor effect on p21 expression. Together, these results are consistent with our previous observation that p21 protects HCT116 wild-type cells from γ IR-induced apoptosis,²⁶ and in addition suggest that caspase-2 modulates DNA damage responses also independently of its catalytic activity by positively regulating p21 expression.

Caspase-2 is commonly required for DNA damage-induced p21 expression.

To verify that the loss of caspase-2 sensitizes HCT116 wild-type cells toward γ IR-induced apoptosis via suppression of p21, we compared the effects of the caspase-2 knockdown on apoptotic responses of wild-type and p21-deficient HCT116 cells. Silencing of caspase-2 increased the amount of γ IR-induced caspase-3-like DEVDase activity in wild-type cells to a comparable level detected in untransfected γ IR-treated p21^{-/-} cells (Fig. 3A). Irradiated p21^{-/-} cells on the other hand were not further radio-sensitized by this measure (Fig. 3A), although a comparable caspase-2 knockdown was achieved as in wild-type cells

(Fig. 3B). These data demonstrate that suppression of p21 is an integral part of the mechanism by which caspase-2 deficiency increases the radio-sensitivity of HCT116 wild-type cells.

To determine whether caspase-2-dependent p21 expression is confined to γ IR-induced signaling, we treated cells with the topoisomerase-II inhibitor etoposide that also resulted in an efficient processing of procaspase-2 (Fig. 3B). Consistently, the absence of caspase-2 in etoposide-treated wild-type cells was also accompanied by a marked reduction of p21, whereas expression of p53 and Puma remained unaffected (Fig. 3B). However, despite the significant reduction in p21, only a marginal increase of DEVDase activity was observed in etoposide-treated caspase-2-depleted wild-type cells when compared with similarly treated untransfected cells or with cells transfected with a control siRNA (Fig. 3C). The failure of p21 to affect etoposide-induced apoptosis is most likely explained by our observation that wild-type and p21^{-/-} HCT116 cells display similar apoptosis sensitivities toward various chemotherapeutic drugs including etoposide (not shown), suggesting that p21 is not sufficient to protect the cells under those conditions.

Having established the consequences of a caspase-2 loss in wild-type and p21^{-/-} HCT116 cells exposed to γ IR and etoposide, we analyzed whether this applies also to other cell types. Therefore, MCF-7/Casp3 breast carcinoma cells that like HCT116 wild-type cells also undergo senescence upon exposure to γ IR,²⁷ were transfected with control or caspase-2 siRNAs. Also in these cells, procaspase-2 was readily processed following γ IR exposure (Fig. 4A) despite their apoptosis resistance toward this treatment.²⁷ More importantly, they too displayed an almost complete loss of the γ IR-induced p21 expression following caspase-2 knockdown, whereas expression of p53 and PUMA remained unchanged (Fig. 4A). Similar results were obtained with etoposide-treated MCF-7/Casp-3 cells (Fig. 4C) or when we exposed caspase-2-depleted primary normal human dermal fibroblasts (NHDF) to γ IR (Fig. 4E). In contrast to wild-type HCT116 cells, however, knockdown of caspase-2 could not alter

their apoptosis susceptibilities (Fig. 4B, D and data not shown), suggesting that other mechanisms besides p21 determine their response toward these treatments. Nevertheless, our results demonstrate that expression of p21 commonly requires the presence of caspase-2.

Caspase-2 is essential for DNA damage-induced checkpoint control.

Next we analyzed the proliferative capability of HCT116 cells in the presence and absence of caspase-2. Wild-type and p21-deficient HCT116 cells were transfected with control or caspase-2 siRNA and incubated for four hours with BrdU two days following their exposure to γ IR in order to label cells actively synthesizing DNA. As expected, regardless of the presence or absence of caspase-2, p21-deficient cells were unable to undergo γ IR-induced cell cycle arrest, as similar numbers of BrdU-positive cells were detected in irradiated and non-irradiated samples (Fig. 5A). In contrast, untransfected or control siRNA-transfected wild-type cells displayed significantly lower numbers of BrdU-stained cells post γ IR (Fig. 5A), demonstrating that the observed cell cycle arrest of wild-type cells depends mainly on the presence of p21. Consistently, diminishing p21 levels by the caspase-2 siRNA increased the number of irradiated wild-type cells that are capable of incorporating BrdU (Fig. 5A).

Remarkably, similar to irradiated p21-deficient cells, irradiated wild-type cells expressing the caspase-2 siRNA harbour extremely aberrant shaped nuclei that are characteristic for cells lacking an important cell cycle checkpoint and hence, try to enter mitosis despite an extensive damage to their DNA (Fig. 5B). As such an abnormal nucleus structure was not observed in irradiated wild-type cells that were left untransfected or transfected with the control siRNA (Fig. 5B), these data demonstrate that caspase-2 is crucial for an orderly executed stress response pathway upon DNA damage.

Caspase-2 modulates p21 expression at the translational level.

Several mechanisms could account for the observed decrease of p21 protein following knockdown of caspase-2 including degradation by the proteasome, cleavage by proteases including caspases or transcriptional repression of the CDKN1A gene encoding p21.^{20,23,28} Therefore, we first asked whether protease-mediated cleavage of p21 contributes to the observed loss. However, irradiating caspase-2-depleted wild-type cells in the presence of the pan-caspase inhibitor Q-VD-OPh could not prevent p21 decrease (Fig. 6A), demonstrating that this event is not caused by caspase cleavage. In addition, Q-VD-OPh failed to inhibit procaspase-2 processing in irradiated control cells, and was completely ineffective in reducing γ IR-induced p21 levels in untransfected wild-type cells, or in cells transfected with the control siRNA (Fig. 6A). Similar results were obtained with z-VDVAD-fmk (not shown), another caspase inhibitor preferentially targeting caspase-2. Based on the effectiveness of Q-VD-OPh and z-VDVAD-fmk to completely suppress caspase-3 processing and activity (Suppl. Fig. 2, and data not shown) and our finding that a catalytically inactive caspase-2 mutant substantially increased p21 expression (Fig. 2D), these data strongly suggest that caspase-2 modulates p21 expression independently of its enzymatic activity.

Next we employed the proteasomal inhibitor MG-132 that due to its high cytotoxicity in HCT116 cells was only applied for a maximum of eight hours post γ IR, a time point clearly sufficient to impair p21 expression in the absence of caspase-2 (Fig. 6B; compare lanes 2/6 with 10). Although MG-132 caused, as expected, a rapid accumulation of p53 and p21 even in the absence of γ IR, it was unable to restore p21 protein levels of non-irradiated and irradiated caspase-2 knockdown cells to those observed in control siRNA-transfected or untransfected cells (Fig. 6B, compare lanes 3/4 and 7/8 with 11/12). Similar results were obtained using other inhibitors targeting either the proteasome (calpain inhibitor-I), calpains and cathepsins (calpain inhibitor-II), or interfering with lysosomal acidification such as ammonium chloride

(not shown). Thus, neither caspases nor any other proteases investigated are involved in the reduction of p21 following caspase-2 knockdown.

Therefore, we analyzed whether p21 transcription is influenced by the presence or absence of caspase-2. However, performing Real-Time-PCR analyses for p21 and Puma mRNA, no significant caspase-2-dependent differences were observed in their control or γ IR-induced expression levels. Although both mRNA species were efficiently upregulated in wild-type cells exposed to γ IR, the knockdown of caspase-2 did not interfere with these processes (Fig. 6C). Therefore, our data argue against a direct off-target effect of the caspase-2 siRNAs on the p21 mRNA and additionally imply that caspase-2 controls p21 expression at the translational level.

As sequences in the 5'- and 3'-untranslated regions (UTR) are important regulators of protein translation,²⁹ wild-type cells were transfected with Flag-tagged p21 cDNA constructs containing either 5'- or 3'-UTR sequences or both or with a construct that only consists of the p21 coding region. Remarkably, although the two Flag-p21 proteins harbouring 3'-UTR sequences were less well expressed than the p21 constructs that contain no UTRs or only the 5'-UTR, knockdown of caspase-2 further compromised their expression in stressed and unstressed cells (Fig. 6D). In contrast, expression of p21 constructs that lack both UTRs or only include the 5'-UTR was not affected in either condition and remained unaltered even in the absence of caspase-2. Obviously, when compared to the endogenous p21, expression of the exogenously expressed Flag-p21-3'-UTR construct was less affected by the depletion of caspase-2. This is most likely due to an important technical difference in the setup of these two experiments. Whereas the endogenous p21 was only induced (e.g. by γ -IR) at a time point at which caspase-2 was already depleted from the cells (48 hours post transfection with the caspase-2 siRNA), the exogenous Flag-p21-UTR constructs were simultaneously cotransfected with the caspase-2 siRNA, as two individual successive transfections proved to

be extremely detrimental to the cells. As the siRNA-mediated knockdown of caspase-2 requires up to 48 hours, this cotransfection procedure denotes that transcription and translation of the exogenous Flag-p21 constructs will be initially supported by the presence of caspase-2. Thus, expression of exogenous Flag-p21 will only be impaired at a later time point at which caspase-2 is sufficiently down regulated by the siRNA. Furthermore, together with the observation that depletion of caspase-2 resulted also in a severe reduction of luciferase activity when the p21 3'-UTR was cloned downstream of the luciferase cDNA⁴² (Fig. 6E), our results provide strong evidence that caspase-2 controls p21 expression at the translational level by an as yet unknown mechanism that involves 3'-UTR sequences of p21.

p53 critically determines the role of caspase-2 in DNA damage responses.

Caspase-2 can be recruited to three functionally different multi-protein complexes; the RAIDD-PIDDosome,¹⁶ the DNA-PKcs-PIDDosome,¹⁴ and to a complex consisting of RIP and TRAF2.¹⁹ To elucidate whether caspase-2 requires association with any of these complexes in order to regulate p21 expression in wild-type cells, we silenced a specific component of each using siRNAs. Although a sufficient knockdown was achieved for DNA-PKcs, RIP and RAIDD (Fig. 7A), none of these prevented processing of procaspase-2 (Fig. 7B), or resulted in a decrease of p21 as it was observed following caspase-2 knockdown (Fig. 7A). Consistently, only irradiated wild-type cells transfected with caspase-2 siRNA showed an increase in DEVDase activity (Fig. 7C), indicating that none of the known complexes are involved in this novel function of caspase-2.

Intriguingly, a completely different picture emerged when we performed similar experiments with cells lacking p53. In sharp contrast to the responses of irradiated wild-type and p21-deficient HCT116 cells, caspase-2 knockdown rendered p53-deficient cells resistant toward γ IR-induced apoptosis and significantly inhibited DEVDase activity (Fig. 8B). Moreover, siRNA-mediated inhibition of RAIDD expression, but not that of RIP or DNA-

PKcs (Fig. 8A), resulted in a similar reduction of γ IR-induced DEVDase activity in these cells as observed following caspase-2 knockdown (Fig. 8B). Together with the observation that only RAIDD deficiency, but not that of RIP or DNA-PKcs prevented processing of procaspase-2 in p53-deficient cells (Fig. 8A), these data indicate that in the absence of p53, caspase-2 signals apoptosis most likely via formation of the RAIDD-PIDDosome.

DISCUSSION

We describe an unprecedented role of caspase-2 as an essential cofactor for the efficient DNA damage-induced expression of the CDK inhibitor p21 that is evident in different cell types independently of the kind of DNA damage applied. This function might allow caspase-2 to participate in diverse signaling pathways modulated by p21 including cell cycle control, gene transcription, DNA repair, differentiation, and apoptosis.²⁰⁻²² Consistently, we observed that the diminished p21 protein pool due to the knockdown of caspase-2 resulted in an increased proliferation rate of irradiated HCT116 wild-type cells, a phenomenon recently reported also with caspase-2-deficient MEFs.¹⁵

Our data also suggest that it is the p21-modulating capability, rather than its apoptosis-inducing potential, that constitutes the primary function of caspase-2, at least within the cellular context examined here. This assumption is based on the observation that knockdown of caspase-2 was not sufficient to protect several p53-expressing cells from DNA damage-induced apoptosis. Even the radio-sensitizing effect observed in caspase-2-depleted wild-type HCT116 cells is probably not caused by a direct influence of caspase-2 on apoptosis signaling, as it rather appears to be a consequence of p21 downregulation. As this sensitization occurs only in cells susceptible to the anti-apoptotic function of p21 such as irradiated HCT116 wild-type cells,²⁶ our data are consistent with a previous report demonstrating that p21 is not the only determinant in stress-induced p53 responses.³⁰ Surprisingly, however, and in contrast to other reports demonstrating a p53 requirement for caspase-2 activation and apoptosis induction,^{31,32} the pro-apoptotic function of caspase-2 unveiled in our study only upon loss of p53. Thus, our data suggest that caspase-2 is additionally part of an apoptotic backup system that signals apoptosis only in the absence of a functional p53 pathway. Although cell type and stimulus-dependent differences clearly contribute to this controversial issue,³¹ our view is further supported by the observation that knockdown of RAIDD, but not that of RIP or DNA-PKcs, successfully abrogated DNA

damage-induced processing and signaling of caspase-2, however, only in p53-deficient HCT116 cells. On the other hand, DNA damage-induced caspase-2 processing and modulation of p21 expression was not affected in wild-type cells by knockdown of any of these components suggesting the existence of additional caspase-2-activating platforms. Consistently, although caspase-2 was found by others to be associated with the RAIDD-PIDDosome even in unstressed wild-type and p53^{-/-} HCT116 cells, silencing of RAIDD and PIDD also failed to prevent caspase-2 activation and apoptosis in 5-FU-treated HCT116 wild-type cells.³¹ Thus, further studies are required to shed some light on this unresolved issue.

In this context, the most important questions remaining are: how does caspase-2 affect expression of p21 and does this process require a processed and active caspase-2 enzyme? A variety of mechanisms known to regulate p21 expression at the protein and DNA/RNA level have been elucidated in recent years.^{20,23,24} However, post-translational regulation could be excluded here, as various inhibitors targeting proteasomal or other proteases could not restore p21 levels following caspase-2 knockdown. Also two different caspase inhibitors (Q-VD-OPh, z-VDVAD-fmk) were despite their effectiveness in completely blocking γ IR-induced caspase-3 processing and activity in p53-deficient cells (Suppl. Fig. 2 and data not shown), unable to prevent loss of p21. Combined with our finding that overexpression of a catalytically inactive caspase-2 mutant was sufficient to increase p21 expression in unstressed wild-type cells, these data strongly suggest that the enzymatic activity of caspase-2 is dispensable for this event. A similar conclusion was reached with regard to its processing, as impaired p21 expression as a consequence of caspase-2 depletion was already observed eight hours post γ IR, a time point at which the decrease of pro-caspase-2 was not even evident in irradiated control cells. However, in regard to this, it is presently not entirely clear whether the observed γ IR-induced decrease of pro-caspase-2 reflects indeed its processing, as both caspase inhibitory peptides neither compromised this process nor the concomitant generation

of mature caspase-2 fragments (Suppl. Fig. 2). Using two different caspase-2 antibodies, the mature large caspase-2 subunit was only generated in irradiated p53- and p21-deficient HCT116 cells, whereas the appearance of a fragment probably resembling the processed prodomain was also detected in wild-type cells. As this fragment might have also arisen from an alternative splicing event,³³ it remains possible that the decrease of caspase-2 in wild-type cells does not reflect its processing, but rather a p53-mediated suppression as reported recently.³⁴ However, Real-Time PCR analyses revealed comparable amounts of caspase-2 mRNA transcripts in unstressed and irradiated wild-type and p53-deficient HCT116 cells (not shown) clearly arguing against this possibility.

While therefore a direct intervention of caspase-2 with p53-mediated p21 transcription seemed reasonable to assume, particularly in view of the observed interaction of p53 with caspase-2 (not shown), irradiated caspase-2-depleted cells exhibited only a slight decrease in p21 mRNA transcripts. Together with our finding that the lack of caspase-2 affects exogenous expression only of those p21 constructs containing 3'-UTR sequences, these results clearly argue against a direct effect of caspase-2 on p21 transcription. In addition, as expression of these 3'-UTR-containing p21 constructs is even severely compromised in the presence of caspase-2, we rather favour a model in which caspase-2 controls p21 expression at the translational level involving 3'-UTR sequences. As caspase-2 does not harbour an obvious RNA-binding domain, we would like to suggest an indirect mechanism by which caspase-2 affects p21-mRNA translation, perhaps via association with RNA-binding proteins or modulation of related pathways. Thereby, we can surely exclude participation of certain RNA-binding proteins such as members of the Hu family that were shown to interact and stabilize p21 mRNA.³⁵ Also CUGBP1 and calreticulin, two RNA-binding proteins that promote and inhibit p21 translation are most likely not involved, as they compete with each other for the binding to the same nucleotide sequence within the 5'-region of the p21 mRNA.³⁶ Our results leave open, however, the possibility that caspase-2 modulates the

function of a different class of RNA-binding proteins known to stimulate or inhibit mRNA translation without interfering with their stabilities. For instance, binding of musashi or the heterogeneous nuclear ribonucleoprotein K (hnRNP-K) to the 3'-UTR of the p21 mRNA was shown to efficiently inhibit its translation.^{37,38} However, whether these or any other RNA-binding proteins are involved in the caspase-2-modulated p21 expression remains to be investigated.

Another possible mechanism by which caspase-2 may modulate p21 translation involves repression of certain microRNAs known to prevent p21 mRNA translation without interfering with its stability.³⁹ Although there might exist an almost overwhelming number of at least 100 microRNAs that theoretically could target the p21 mRNA (as predicted from microRNA.org), we have analyzed the expression levels of miR-17, miR-20a, and miR-106b, because these were not only shown to efficiently block p21 mRNA translation,⁴⁰⁻⁴² but were also found to be repressed by p53.⁴³ However, expression of these microRNAs did not change 16 hours following exposure of HCT116 wild-type cells to γ IR, and also remained unaltered in the presence or absence of caspase-2 (not shown). In contrast, the p53-inducible miR-34a that is involved in the induction of apoptosis and senescence⁴⁴ was found upregulated following γ IR, however, in a caspase-2-independent manner (not shown). Although these observations clearly argue against an involvement of these microRNAs in the caspase-2-dependent expression of p21, the possibility remains that other microRNAs contribute to this process.

In summary, we have uncovered an all new function for caspase-2 as an essential cofactor for DNA damage-induced p21 expression. Certainly, this finding entails far-reaching implications, as the translational regulation of p21 enables caspase-2 to participate in the diverse signaling processes controlled by this CDK inhibitor. Now it will be challenging to identify the components involved and to elucidate the mechanism of how exactly caspase-2 regulates p21 translation.

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MATERIALS AND METHODS

Cell lines, reagents and antibodies. HCT116 wild-type cells and their checkpoint-deficient variants (p53^{-/-} and p21^{-/-}) were maintained in McCoy's 5A medium (PromoCell, Heidelberg, Germany), whereas MCF-7/casp3 cells were cultured in RPMI 1640 (PAA Laboratories, Linz, Austria) in the presence of 400 µg/ml neomycin. The primary normal human dermal fibroblasts (NHDF) were obtained from PromoCell and were cultured in DMEM low glucose supplied with 1% MEM nonessential amino acids solution and 0.1 mM β-mercapto-ethanol. Only passages below P20 were used. All media were supplemented with 10% heat-inactivated fetal calf serum, 10 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (PAA Laboratories). The pan-caspase inhibitory peptide Q-VD-Oph (Q-Val-Asp-CH₂-O-Ph) as well as the caspase-2 inhibitory peptide z-VDVAD-fmk (z-Val-Asp-Val-Ala-Asp-fluoromethyl-ketone) were from MP Biomedicals (Irvine, CA, USA). The fluorometric caspase-3 substrate DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) was from Biomol (Hamburg, Germany). The polyclonal antibodies against caspase-3 and caspase-9 were from R&D Systems (Wiesbaden, Germany) and from Cell Signaling Technology (Danvers, MA, USA), respectively. The rat caspase-2 mAb was from Alexis Biochemicals (Lausen, Switzerland). The p53 monoclonal Ab-6 antibody and the polyclonal antibody for PUMA were from Calbiochem (Bad Soden, Germany), whereas the mAbs recognizing p21 and RIP were from BD Biosciences (Heidelberg, Germany). The mAb towards RAIDD was from MBL International (Woburn, MA, USA). From Santa Cruz (Heidelberg, Germany) was the polyclonal antibody recognizing the catalytic subunit of DNA-PK. The actin mAb, the nuclear stain 4',6-diamidino-2-phenylindole (DAPI), the topoisomerase II inhibitor etoposide, the two calpain inhibitors I (ALL-N) and II (ALL-M) and the protease inhibitors PMSF, aprotinin, leupeptin and pepstatin were from Sigma-Aldrich (Deisenhofen, Germany). From Enzo Life Sciences (Biozol; Eching, Germany) we

obtained the proteasome inhibitor MG-132, and the peroxidase-labeled secondary antibodies were from Promega GmbH (Mannheim, Germany).

Treatment of cells and measurement of cell death. Cells were exposed to γ IR (routinely 20 Gy at 200 kV) using a Gulmay RS 225 X-ray system from IsodoseControl (Bochum, Germany) or were treated with etoposide (50 μ M) for the indicated times. Cell death was assessed either microscopically or by determination of lactate dehydrogenase (LDH) activity in supernatants of 10^5 cells according to the protocol of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The values obtained are given in arbitrary units (AU).

Preparation of cell extracts and Western blotting. Total cell extracts were prepared in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% NP-40) containing protease inhibitors as described.²⁶ Protein concentrations were determined with the BioRad protein assay, followed by separation of the extracts in SDS-polyacrylamide gels and electroblotting onto polyvinylidene difluoride membranes (Amersham Biosciences, Braunschweig, Germany). Following antibody incubation, the proteins were visualized by enhanced chemiluminescent staining using ECL reagents (Amersham Biosciences).

Fluorometric determination of caspase-3-like-activity (DEVDase assay). For the detection of caspase-3-like activities, 50 μ g of the cell extracts were incubated for 3 to 4 hours with 50 μ M of the caspase-3 substrate DEVD-AMC in 200 μ l buffer containing 50 mM HEPES (pH 7,4), 100 mM NaCl, 10% sucrose, 0.1% CHAPS and 10 μ M DTT. The release of fluorogenic AMC was measured at an excitation wavelength of 346 nm and an emission wavelength of 442 nm using an Infinite M200 microplate reader (Tecan, Langenfeld, Germany). The detected fluorometric signal that directly correlates to the caspase activity in the cell extracts is expressed in arbitrary units (AU).

Plasmids and generation of the different p21-UTR constructs: The plasmids encoding Flag-tagged wild-type caspase-2 or an inactive mutant (C320G) (pEF-3x-Flag-Casp2-WT and –MUT) were generous gifts from A. Villunger (Innsbruck, Austria). The plasmids pCEP-Waf1⁴⁵ and pMT5-Flag-21-WT⁴⁶ were purchased from Addgene (Cambridge, MA, USA) and served as templates for cloning of the different p21-UTR constructs that all contain a N-terminal Flag epitop (see below). The open reading frame of Flag-p21 was amplified from the pMT5-Flag-p21-WT plasmid using primers that harbour BamHI and EheI restriction sites at the 5'-end and SacII and EcoRI sites at the 3'-end. Following digestion with BamHI and EcoRI, the amplified sequence (~ 650 bp) was cloned into the pcDNA4-Myc-His-A vector (Invitrogen) yielding the pcDNA4-Flag-p21 plasmid. The 5'-UTR of p21 (~ 80 bp) was amplified from the pCEP-WAF1 plasmid using primers with a HindIII-site at the 5'-end and an EheI-site at the 3'-end. After digestion with HindIII and EheI, this amplificate was cloned into the pcDNA4-Flag-p21 plasmid resulting in the pcDNA4-5-UTR-Flag-p21 plasmid. Similarly, the 3'-UTR of p21 (~ 1500 bp) was amplified via PCR from the pCEP-WAF1 plasmid with primers including SacII at the 5'-end and NotI at the 3'-end and subsequently cloned into pcDNA4-Flag-p21 following digestion with these two enzymes, thereby generating the pcDNA4-Flag-p21-3-UTR plasmid. To finally produce a plasmid expressing the full length p21 mRNA, the 3'-UTR was cut out of the pcDNA4-Flag-p21-3-UTR plasmid with SacII and NotI and cloned into the pcDNA4-5-UTR-Flag-p21 plasmid. The generated plasmid was termed pcDNA4-5-UTR-Flag-p21-3-UTR.

Transfection of siRNAs and plasmids. ON-TARGET^{plus} SMARTpool siRNAs were purchased from Dharmacon RNA technologies (Lafayette, CO, USA) and the knockdown was performed according to the instructions of the manufacturer. 24-48 hours post-transfection, cells were harvested and divided equally to receive either no treatment or exposure to γ IR or etoposide. At the indicated time points following DNA damage, cells were harvested and

directly analyzed by Western blotting and by the fluorometric caspase substrate assay for a successful knockdown of the target protein and DEVDase activity, respectively. For the simultaneous transfection of plasmids and siRNAs, the Dharmafect DUO transfection reagent was used according to the manufacturers protocol. Briefly, HCT116 wild-type cells seeded in 6-well plates were transfected with 2 µg plasmid and 200 nmol siRNA using 4 µl Dharmafect Duo in 2 ml antibiotics-free medium. One day after transfection, the cells were trypsinized, divided equally into two wells and directly γ -irradiated or left untreated as a control. One day after irradiation the cells were harvested and analysed by Western Blot.

Real-Time PCR. Total RNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany) according to the protocol of the manufacturer. Reverse transcription was achieved with the High Capacity cDNA Kit from Applied Biosystems (Darmstadt, Germany). Taqman gene expression probes for human p21, Puma and actin mRNA (Applied Biosystems) were employed to analyse their relative expression levels using the 7300 Real-Time PCR system (Applied Biosystems). The actin mRNA served as an endogenous normalization control for every sample. The fold induction of the analyzed RNAs was calculated via the $2^{-(\Delta\Delta Ct)}$ -method thereby normalizing all samples to the level of the analyzed RNA in untransfected control HCT116 wild-type cells.

Luciferase Reporter Assay. Cells were transfected with a vector containing luciferase cDNA with or without the p21-3'-UTR⁴² together with either caspase-2 or control siRNA. Two days after transfection, cells were harvested and divided into two aliquots. Luciferase activities were determined by using the Dual luciferase activity kit (Promega) and a Centro LB 960 luminometer (Berthold Technologie, Bad Wildbad, Germany). For normalization of the

obtained activities, Real-Time-PCR was performed with a specific firefly luciferase mRNA probe (Custom Taqman Assay from Applied Biosystems).

BrdU labeling. Cells actively synthesizing DNA were labeled with the BrdU (5-Bromo-2'-deoxy-uridine) Labeling and Detection Kit I from Roche Molecular Biochemicals according to the manufacturers protocol. In short, following siRNA transfection, cells were seeded on 15 mm coverslips and irradiated. Two days post γ IR, cells were labeled for four hours with BrdU before they were fixed, incubated with a monoclonal antibody recognizing BrdU followed by incubation with a fluorescence-coupled antibody directed against the primary antibody. Total DNA was stained with DAPI before the coverslips were mounted on glass slides. Subsequently, 6-10 representative pictures were taken from every sample using a 20x objective from the Zeiss Axio Observer A1 and the corresponding AxioVision Software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). The number of cells containing green (BrdU-positive) and blue (DAPI) nuclei were counted from all representative pictures taken. Employing this method, 500 to 1,200 blue cells were analysed for every condition in each single experiment.

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LEGENDS TO FIGURES

Figure 1. Loss of p21 radiosensitizes HCT116 wild-type cells towards γ IR-induced apoptosis. (A) Western blot analyses for the status of the indicated proteins in HCT116 wild-type and p21^{-/-} cells that were γ -irradiated and cultured for the indicated days. (B) HCT116 wild-type cells were either left untransfected or transfected with control and p21 siRNAs. Following γ -irradiation, cells were cultured for the indicated days before the extracts were subjected to Western blot analyses. For A and B, blots shown represent results from two and three independent experiments, respectively. (C) Determination of DEVDase activities in HCT116 wild-type cells transfected and treated as described in B and analyzed after the indicated days. Arbitrary units (AU) shown are the mean from three independent experiments +/-SD.

Figure 2. Modulation of p21 expression by caspase-2. (A) Western blot analyses for the status of the indicated proteins in HCT116 wild-type cells that were either left untransfected or transfected with the caspase-2 siRNA before they were exposed to γ IR and cultured for the indicated days. (B) Determination of DEVDase activities in HCT116 wild-type cells that were either left untransfected or transfected with control or caspase-2 siRNAs before they were γ -irradiated and cultured for the indicated days. Please see Fig. 3A and 7C for additional independent results. (C) Cell death assessment of HCT116 wild-type cells that were either left untransfected or transfected with control or caspase-2 siRNAs before γ -irradiation. The release of LDH into their supernatants was determined five days post γ IR. Arbitrary units (AU) shown in B and C are the mean from two independent experiments +/-SD. (D) Western blot analyses demonstrating the influence of overexpression of a Flag-tagged caspase-2 wild-type protein and a catalytically inactive caspase-2 mutant on p21 expression in control and γ -

irradiated wild-type cells. Blots shown in A and D are representative results out of two and three independent experiments, respectively.

Figure 3. DNA damage-induced p21 expression requires caspase-2. (A) Determination of DEVDase activities in HCT116 wild-type and p21^{-/-} cells that were either left untransfected or transfected with the indicated siRNAs before they were γ -irradiated and cultured for two days. (B) Western blot analyses for the status of the indicated proteins in HCT116 wild-type and p21^{-/-} cells that were either left untransfected or transfected with control or caspase-2 siRNAs before they were exposed to γ IR or etoposide. Cells were harvested 36 hours (etoposide) or 48 hours (γ IR) post treatment. Blots shown represent results from two independent experiments. (C) Determination of DEVDase activities in HCT116 wild-type cells that were either left untransfected or transfected with the control or caspase-2 siRNAs before they were exposed to etoposide. Arbitrary units (AU) shown in A and C are the mean from three independent experiments each +/-SD.

Figure 4. Caspase-2 is commonly required for p21 expression. (A, C, E) Western blot analyses for the status of the indicated proteins in MCF-7/Casp3 cells and NHDFs that were either left untransfected or transfected with control or caspase-2 siRNAs before γ -irradiation (A, E) or etoposide treatment (C). Cells were harvested two (E) to three (A) days post γ IR and 24 hours following etoposide. Blots shown represent results from three independent experiments. (B and D) Determination of DEVDase activities in MCF-7/Casp3 cells that were transfected and treated as described in A and C. Arbitrary units (AU) are the mean from three independent experiments +/-SD.

Figure 5. Caspase-2-deficient HCT116 wild-type cells exhibit an increased proliferation rate following γ -irradiation. (A) Determination of the percentage of BrdU-positive HCT116

wild-type and p21^{-/-} cells that were transfected with control or caspase-2 siRNAs before γ -irradiation. BrdU and DAPI stainings were performed two days post γ IR. Data shown are the mean of two independent experiments \pm SD. **(B)** Representative micrographs of HCT116 wild-type and p21^{-/-} cells that were transfected and treated as described in A. Please note that an increased number of irradiated wild-type cells display an aberrant nuclear structure only following knockdown of caspase-2. This phenomenon can be also observed in irradiated p21-deficient cells, however, regardless of whether or not they were transfected with the caspase-2 siRNA.

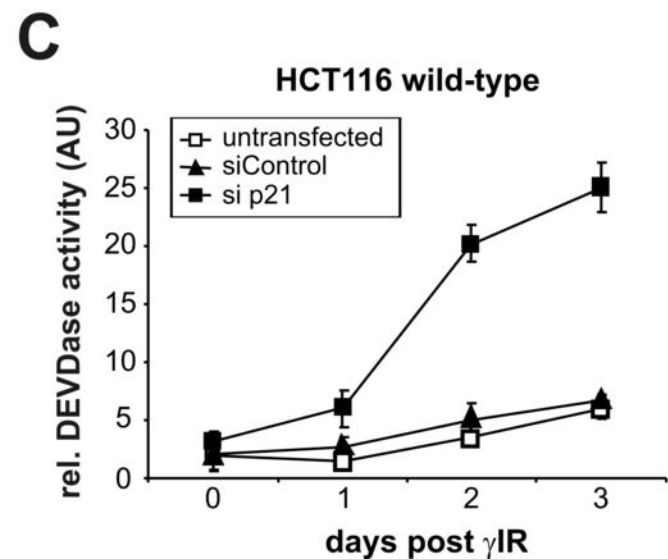
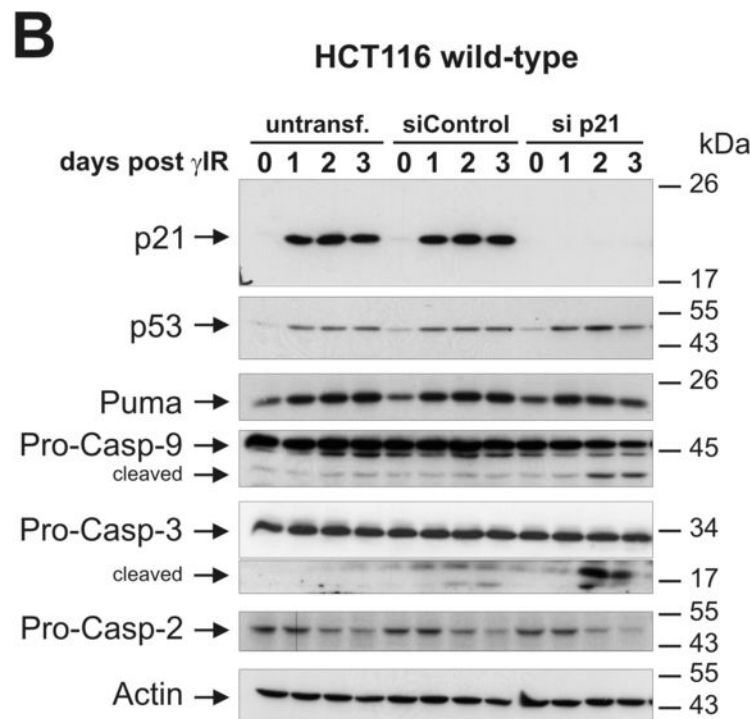
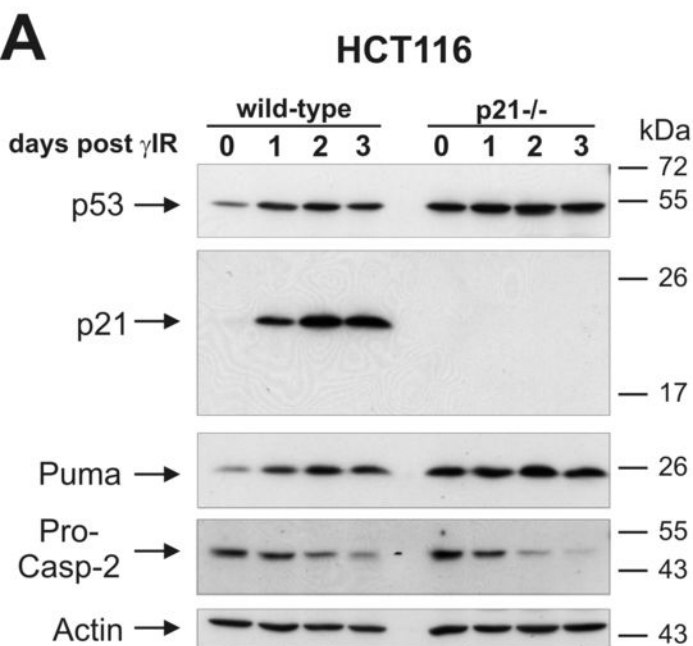
Figure 6. Caspase-2 modulates p21 expression at the translational level. **(A and B)** HCT116 wild-type cells were either left untransfected or transfected with control or caspase-2 siRNAs before they were γ -irradiated in the presence or absence of the pan-caspase-inhibitor Q-VD-OPh (A) or after an 1 hour preincubation with the proteasomal inhibitor MG-132 (B). Cells were harvested 2 days (A) or 8 hours (B) post γ IR and their cellular extracts were analysed by Western blotting for the expression of the indicated proteins. Blots shown represent results from two (A) and three (B) independent experiments, respectively. **(C)** Real-Time-PCR for the determination of the expression levels of the p21 and Puma mRNAs in HCT116 wild-type cells that were either left untransfected or transfected with control or caspase-2 siRNAs before γ -irradiation. Total RNA was isolated 4 hours post γ IR and analyzed with transcript-specific probes from Applied Biosystems. Data shown are the mean of three independent experiments \pm SD. **(D)** Western blot analyses demonstrating the influence of a caspase-2 knockdown on expression of the indicated Flag-tagged p21 constructs containing either 5'- or 3'-UTRs or both in unstressed and irradiated HCT116 wild-type cells. As a control a plasmid encoding only the Flag-p21 open reading frame was used. Representative blots out of three independent experiments are shown. **(E)** Luciferase reporter assay showing the influence of a caspase-2 knockdown on expression of luciferase cDNA constructs with or

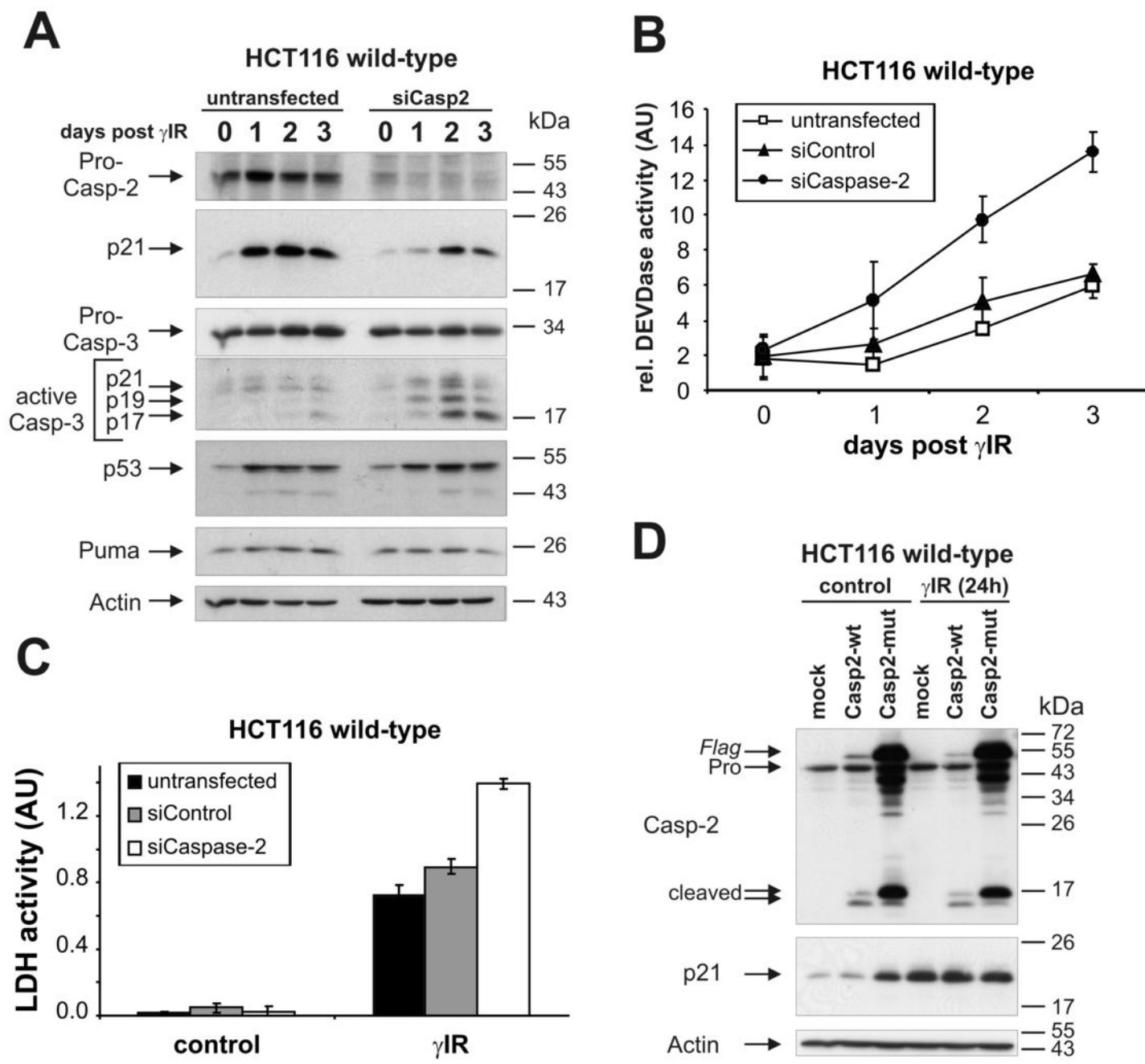
without the p21-3'-UTR. In each sample, the obtained luciferase activity was normalized to the amount of luciferase mRNA. Shown is the percentage of luciferase activities obtained from caspase-2 siRNA-treated cells compared to control siRNA-treated cells. Values are derived from three independent experiments (+/- SD).

Figure 7. Caspase-2 modulates p21 expression independently of known caspase-2-activating complexes. (A) Western blot analyses for the status of the indicated proteins in HCT116 wild-type cells that were either left untransfected or transfected with the indicated siRNAs before γ -irradiation. Cells were analyzed 8 hours post γ IR. Blots shown represent results from three independent experiments. The asterisk denotes a band of unknown origin that was still detectable following DNA-PKcs knockdown serving as an additional loading control. (B) To demonstrate caspase-2 processing in these cells, an event not evident 8 hours post γ IR (see A), HCT116 wild-type cells were treated as in A, but were analyzed for caspase-2 processing three days post γ IR. Similar results were obtained two days post γ IR (data not shown). (C) Determination of DEVDase activities in HCT116 wild-type cells that were treated as described in A. Cells were analyzed 3 days post γ IR. Arbitrary units (AU) shown are the mean from three independent experiments +/-SD.

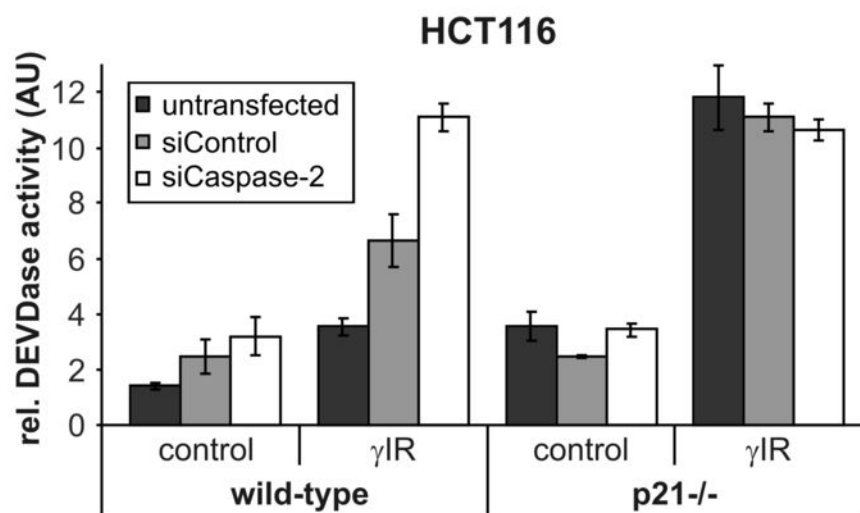
Figure 8. RAIDD and caspase-2 are required for γ -IR-induced apoptosis in p53-deficient cells. (A) Western blot analyses for the status of the indicated proteins in HCT116 p53^{-/-} cells that were either left untransfected or transfected with the indicated siRNAs before they were γ -irradiated. Cells were analyzed 3 days post γ IR. Blots shown represent results from three independent experiments. The asterisk denotes a band of unknown origin (see also figure legend 7A). (B) Determination of DEVDase activities in HCT116 p53^{-/-} cells that were

treated as described in A. Cells were analyzed 3 days post γ IR. Arbitrary units (AU) shown are the mean from three independent experiments \pm SD.

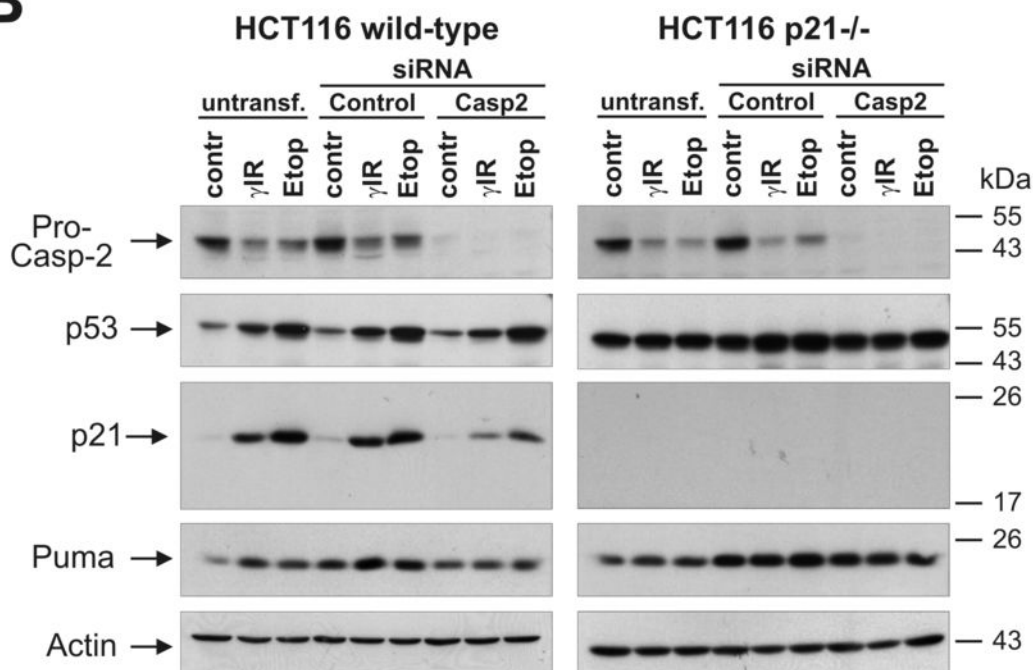




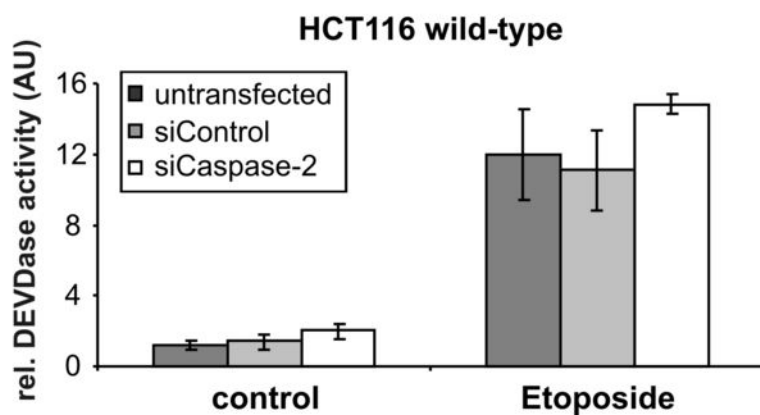
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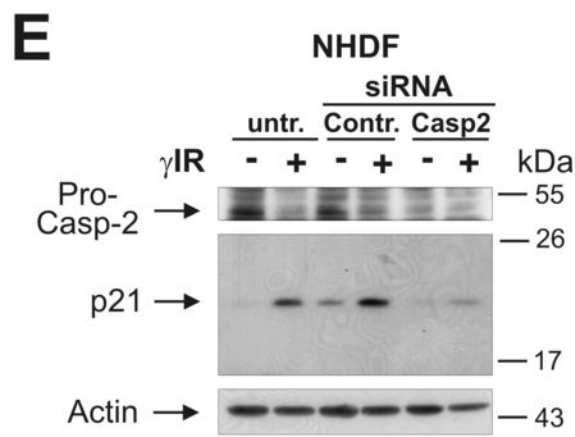
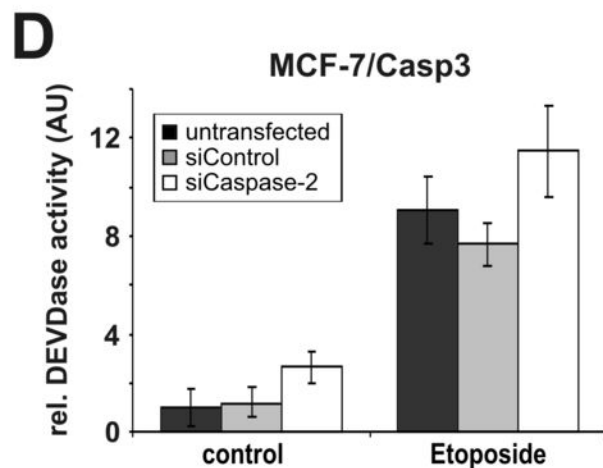
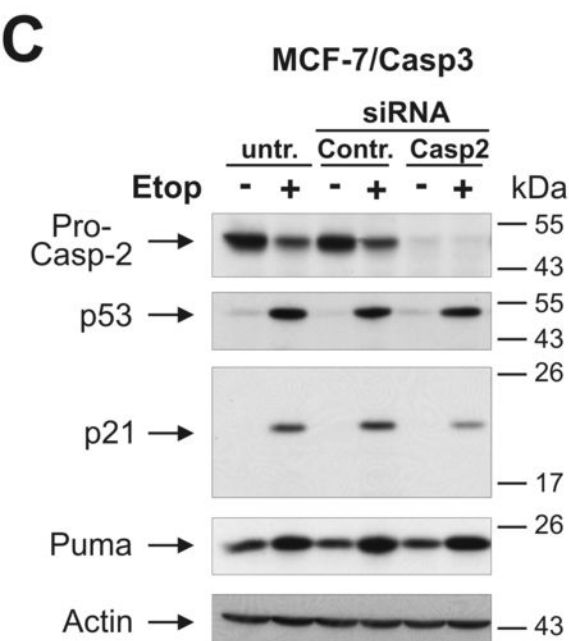
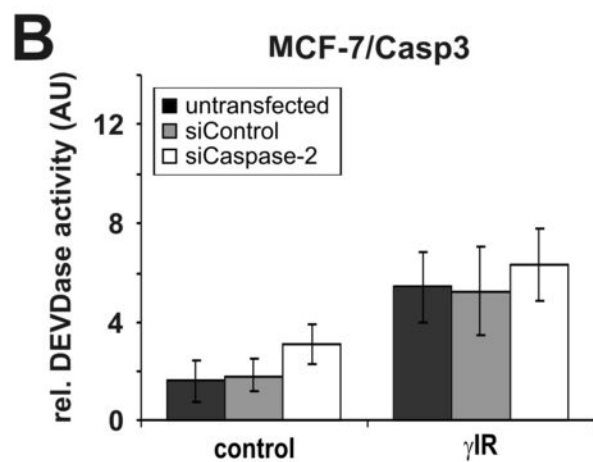
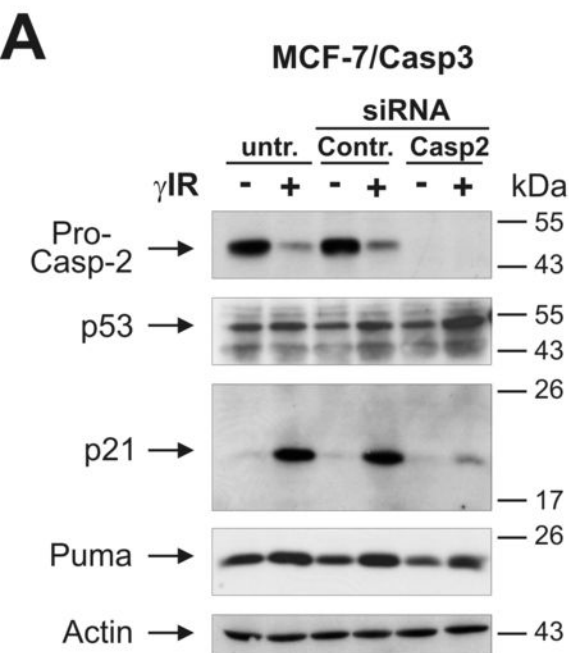


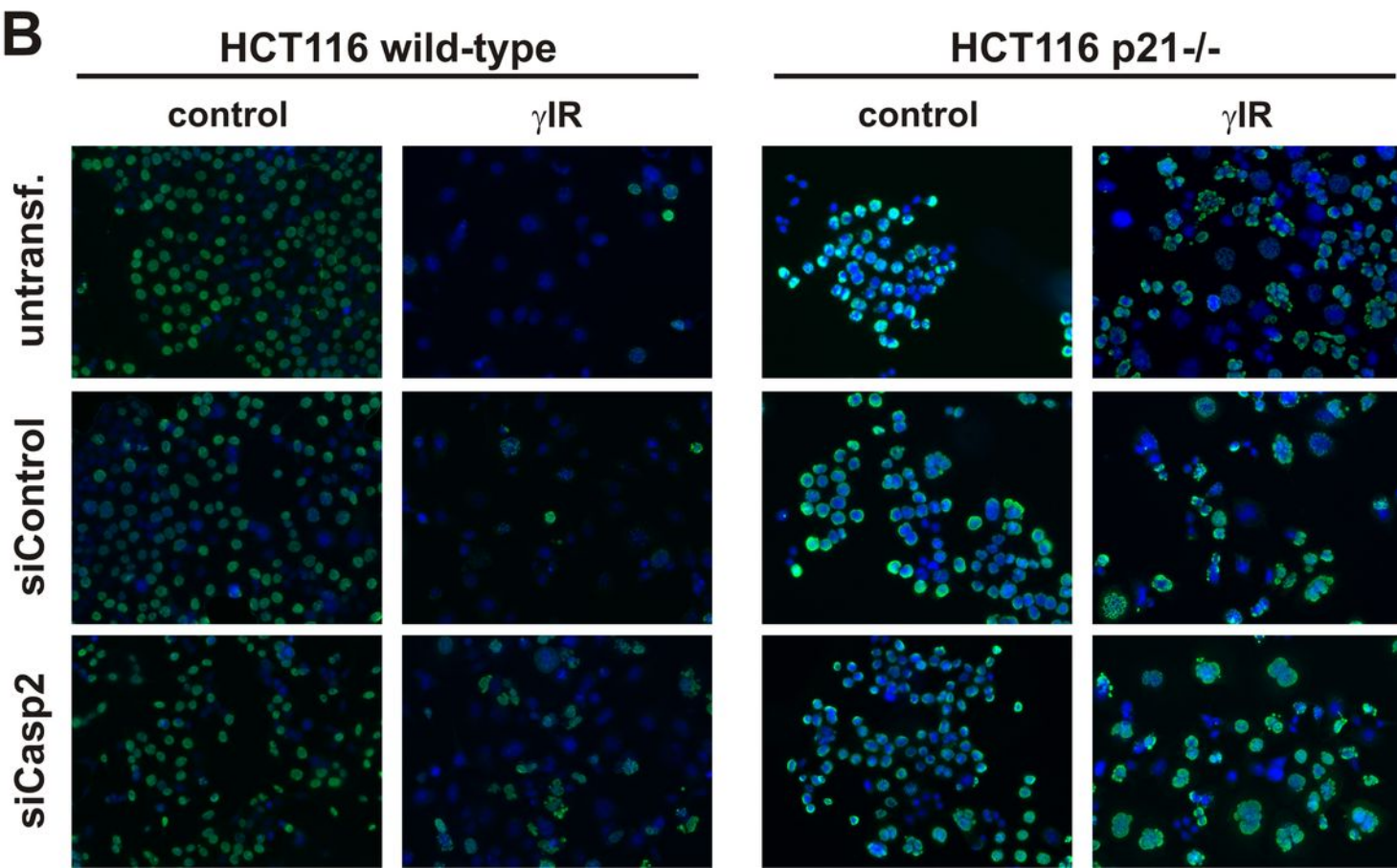
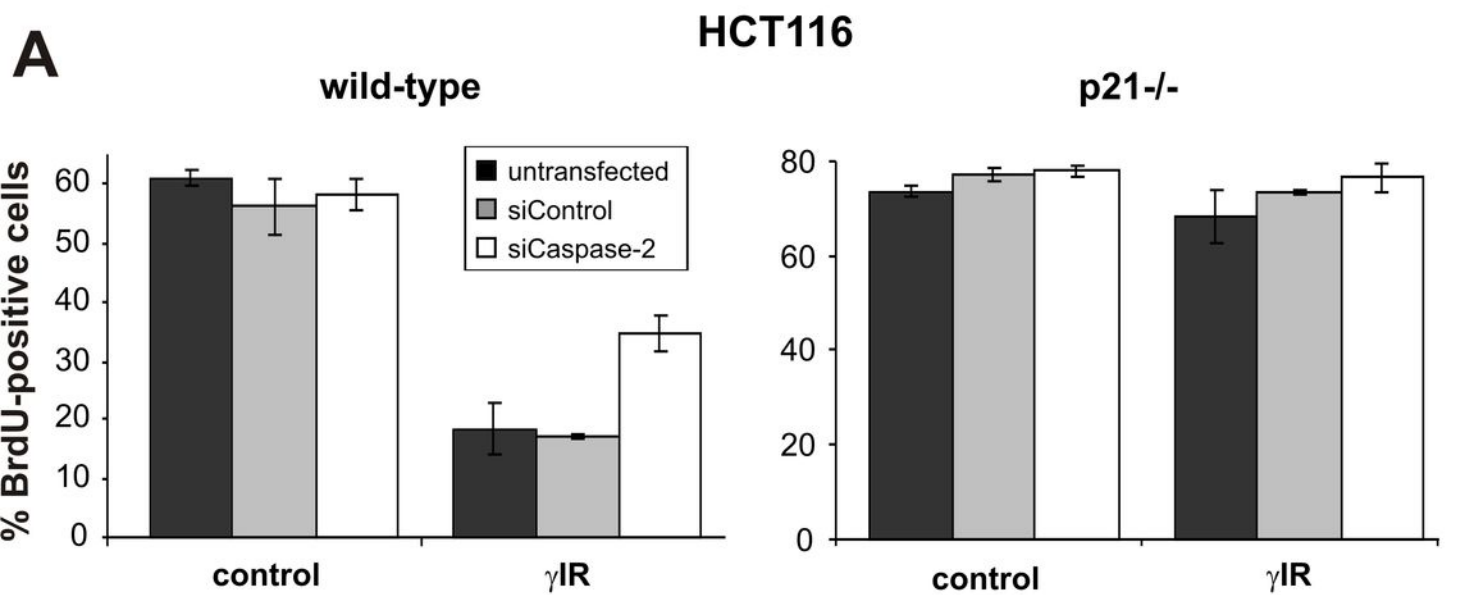
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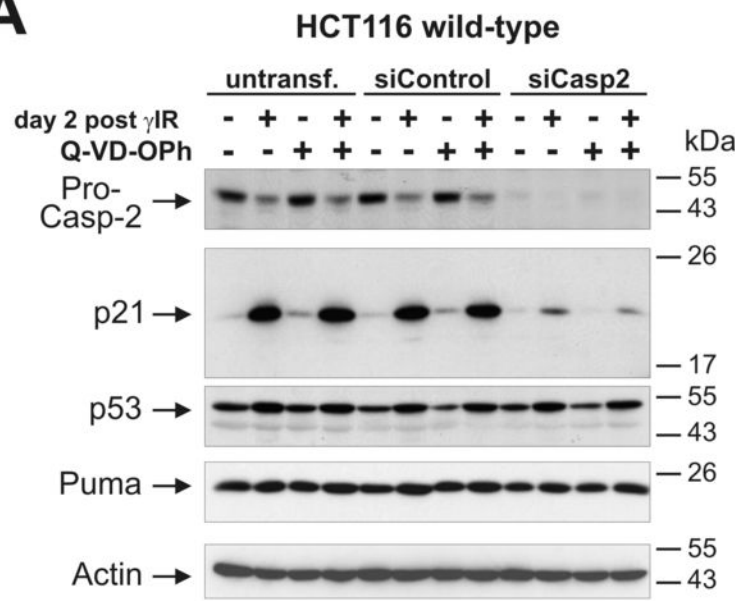
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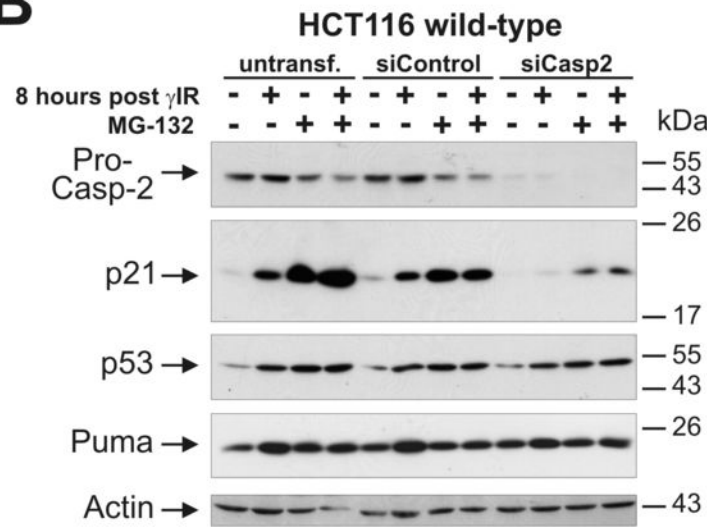




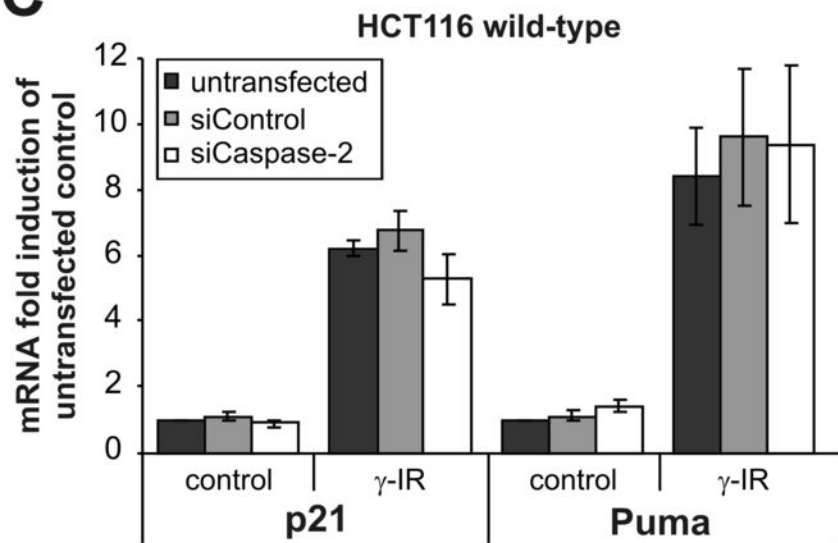
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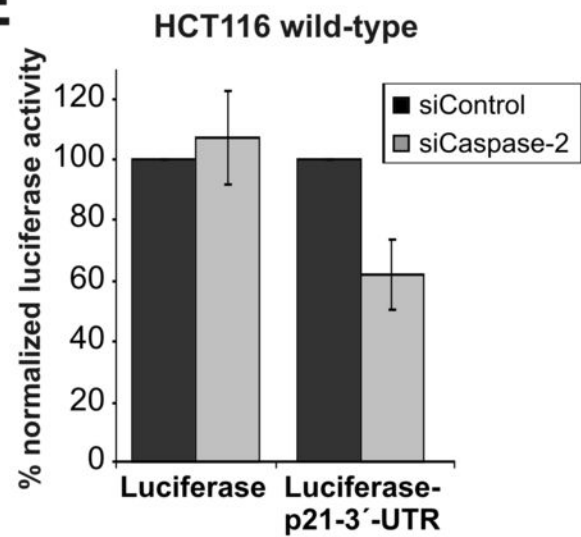
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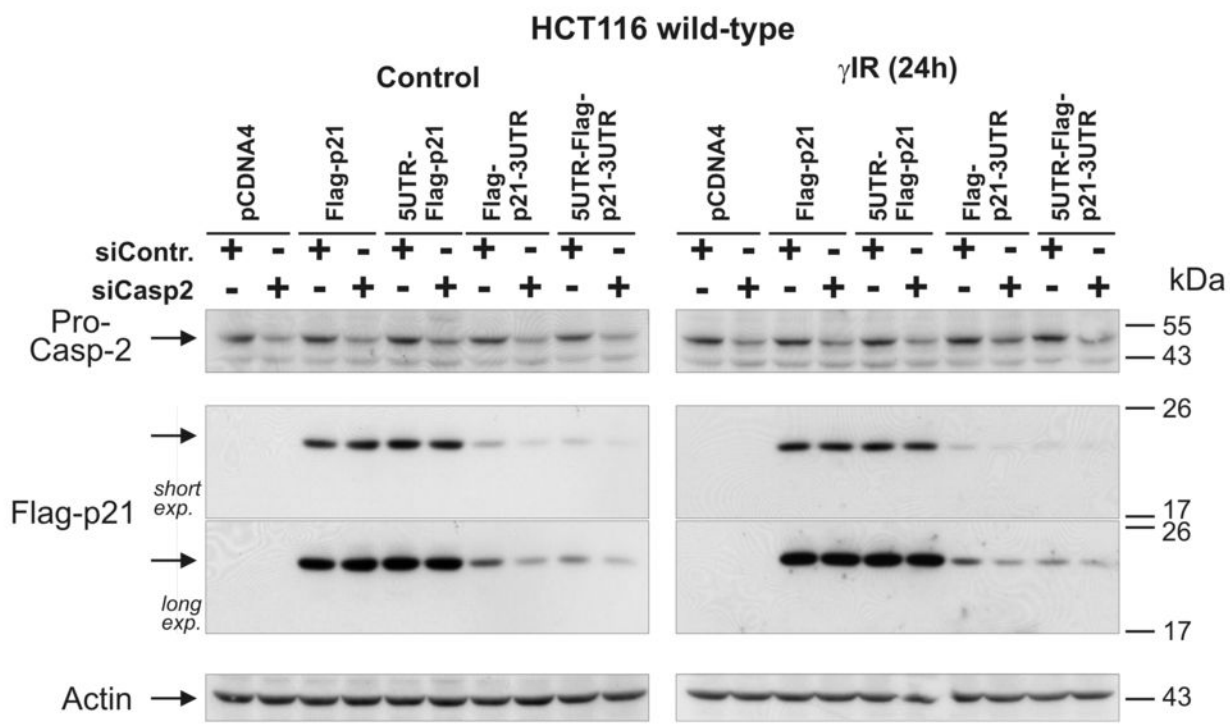
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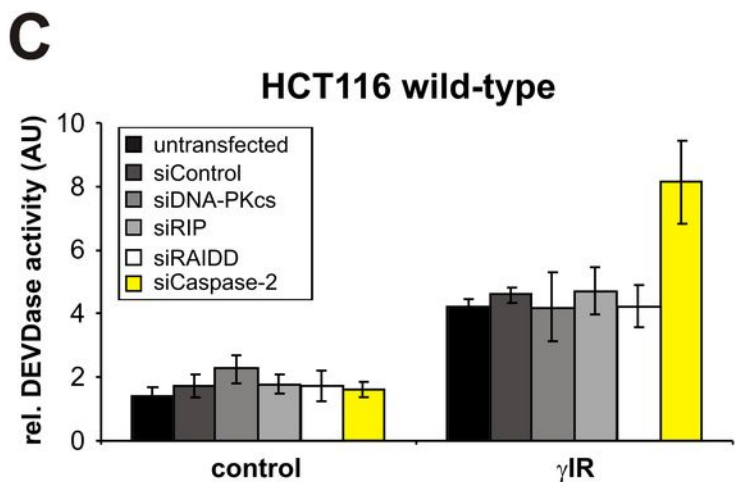
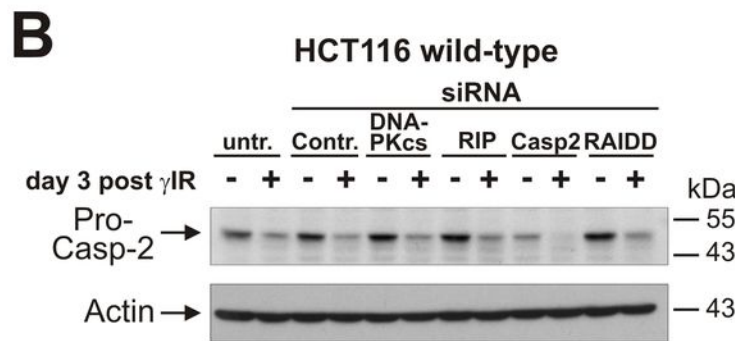
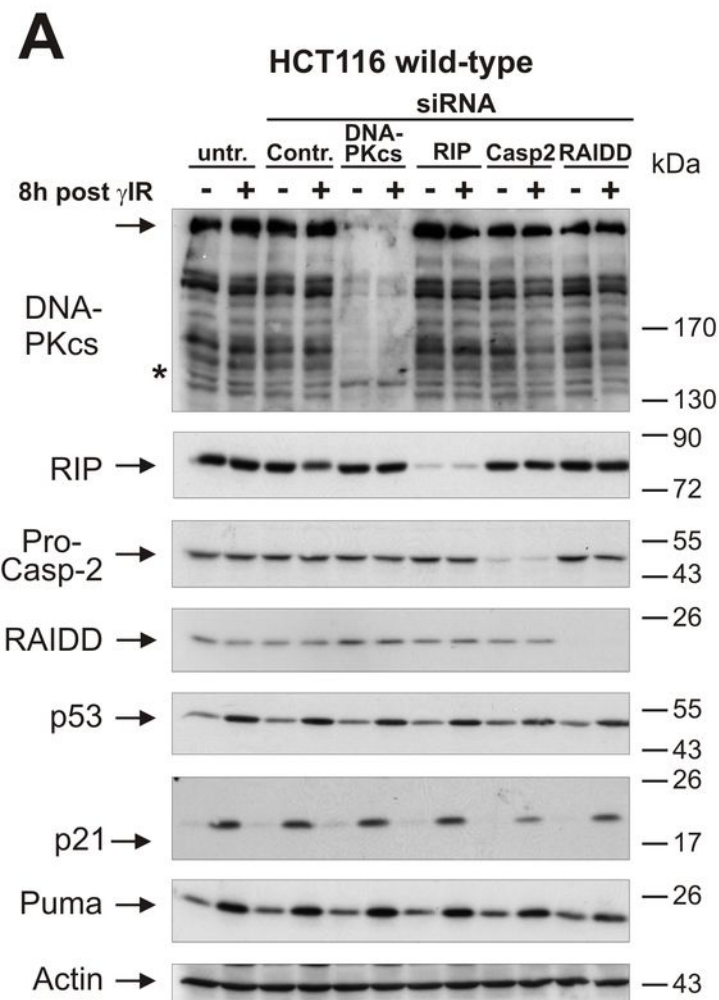


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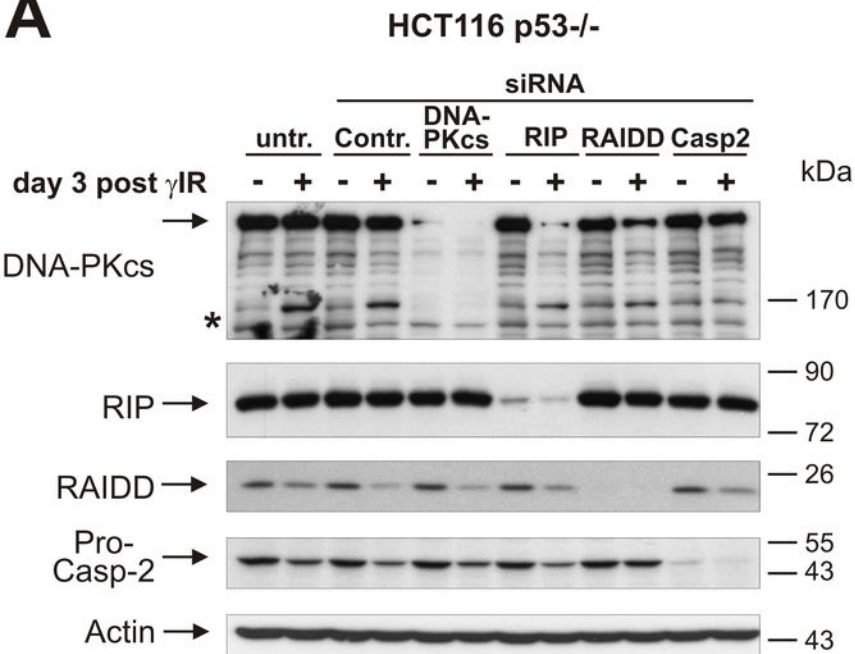


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