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RESEARCH ARTICLE

Isolation of peptides blocking the function of anti-apoptotic Livin protein

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Abstract Livin (ML-IAP) is a cancer-associated member of the inhibitor of apoptosis protein (IAP) family. By yeast two-hybrid screening of a randomized peptide expression library, we isolated short linear peptides that specifically bind to Livin, but not to other IAPs. Intracellular expression of the peptides sensitized *livin*-expressing cancer cells toward different pro-apoptotic stimuli. The bioactive peptides neither showed sequence homologies to Smac-derived IAP inhibitors, nor did they interfere with the binding of Livin to Smac. Intracellular expression of the peptides did not affect the levels or the subcellular distribution of Livin. Growth of *livin*-expressing tumor cells was inhibited in colony formation assays by the Livin-targeting peptides. These findings provide evidence that the targeted inhibition of Livin by peptides represents a viable approach for the apoptotic sensitization and growth inhibition of tumor cells. The inhibitory peptides isolated here could form a novel basis for the development of therapeutically useful Livin inhibitors.

Keywords Cancer · Apoptosis · IAP · Livin · Peptides

Introduction

Resistance toward apoptotic stimuli is a hallmark of cancer cells [1] and is considered to be a major cause for cancer treatment failure in the clinic, since many anticancer agents act through induction of apoptosis [2]. One important mechanism by which cancer cells are believed to acquire apoptosis resistance is overexpression of inhibitor of apoptosis proteins (IAPs) [3].

Livin is a member of the IAP family and is highly expressed in a variety of human neoplasms, but not, or to substantially lower levels, in the corresponding normal tissues [4–6]. Consequently, it has been suggested that the targeted inhibition of Livin may provide a novel therapeutic anticancer strategy [7]. This approach, however, most likely will not selectively affect tumor cells, since there is evidence for Livin expression in specific cells of normal adult tissues. For example, Livin is detectable in testis, thymus, and glomerular mesangial cells, as well as in podocytes, and distal and collect tubule epithelial cells of the kidney, suggesting that Livin also has a function in normal tissues [8, 9].

The *livin* gene is susceptible to efficient silencing by RNA interference (RNAi) [10], which sensitized tumor cells to chemotherapeutic drugs. However, the therapeutic application of small interfering (si)RNAs is still hampered by technical hurdles, which include off-target effects [11] and stimulation of the innate immune response [12]. Off-target effects can result from partial complementarities between the siRNA and a target mRNA, which may mimic interactions with microRNA (miRNA), leading to translational repression or transcript destabilization [13]. Additionally, therapeutic siRNAs face the risk of saturating the endogenous miRNA machinery, which may also lead to unwanted side-effects [14]. Finally, the problem of efficient delivery of therapeutic siRNAs into the organs or cells of interest is still largely unresolved [15].

An alternative approach to specifically interfere with the activity of a potential therapeutic target is its inhibition at

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the protein level. Peptides derived form the IAP inhibitor Smac (second mitochondrial activator of caspases) can block IAPs, thereby exerting anti-tumorigenic effects [16, 17]. Due to their ability to bind to distinct surface regions of a target, inhibitory peptides should provide an important advantage for the validation of therapeutic targets. Specifically, blocking gene expression by RNAi or antisense oligonucleotides will result in the intracellular depletion of the whole protein. In contrast, by inhibiting only distinct protein–protein interactions, peptide-induced perturbations of the molecular network surrounding the target protein are expected to be more subtle and will more closely resemble the effects of a therapeutic agent (e.g., a small molecule inhibitor) targeting the same domain [18]. Furthermore, binding of the peptides to the target can guide the identification of small bioactive molecules with therapeutic potential by displacement screening assays [18].

Here, we identified a series of novel Livin-binding peptides from a randomized peptide expression library. These peptides shared no obvious sequence homologies with the natural Livin inhibitor Smac, and consequently no similarity with any of the previously generated Smac-like peptides or peptidomimetics blocking IAPs [19]. We characterized the binding of the peptides to both known Livin isoforms (Livin α and Livin β), investigated their potential to sensitize *livin*-expressing tumor cells toward apoptosis, analyzed their effects on the levels and intracellular distribution of Livin, and assessed their influence on the growth of *livin*-expressing cells.

Materials and methods

Peptide screening

Yeast-expression vectors encoding Livin α and Livin β proteins fused to the GAL4-DNA-binding domain (GAL4BD) were obtained by subcloning the corresponding coding sequences into pPC97 [20]. Livin β was used as bait for screening. As prey, a peptide-expression library was used, which was generated by fusing randomized 60mer oligonucleotides to the GAL-activation domain (AD) in pADtrx [21], thereby replacing its trx insert. Oligonucleotides contained triplets of the sequence NNK (where N = G, A, T or C; K = G or C) which encode for all 20 amino acids but result in only one stop codon [22]. Screening was performed as previously described, using yeast test strain KF1, which contains three selectable markers of different stringencies [21]. Following selection for growth on adenine-deficient medium (activation of the intermediate stringency *GAL2-ADE2* marker), KF1 transformants were analyzed by replica plating for their ability to also induce the *GAL1-HIS3* (low stringency) and *SPO13-URA3* (high stringency) markers. Peptide-expression vectors from clones, which activated all three marker genes, were rescued and activation of the selectable markers was verified by rescreening. Yeast two-hybrid analyses for binding specificity were performed as reported previously [21], by expressing the human papillomavirus (HPV) type-16 E6 [21], cIAP-1, cIAP-2, XIAP [23], and Survivin from pPC97.

Cell lines, transfections, and plasmids

HeLa, H1299, and MeWo cells were maintained in Dulbecco's modified Eagle's medium (D-MEM), supplemented with 10% fetal calf serum (FCS). Caki-1 cells were grown in RPMI medium, supplemented with 10% FCS. All cell lines were obtained from the DKFZ tumor cell bank and were tested by Multiplex cell Culture Testing, as previously described [24]. Plasmids were transfected by calcium–phosphate co-precipitation [25].

For expression in mammalian cells, individual peptide sequences were fused to a 6-Histidine-9-Arginine (H6R9) tag in pIRESneo (Clontech, Mountain View, CA, USA). This particular tag enables detection of peptide expression with anti-His monoclonal antibodies (H6) and may form a basis for the generation of cell-permeable peptides (R9) [26]. pIRESneo-Smac was obtained by subcloning the Smac cDNA from pACT-Smac [27] into pIRESneo.

Livin β was expressed in mammalian cells from pcDNA3 (Invitrogen, Karlsruhe, Germany). Livin β deletion mutants Livin β 1-189, Livin β 80-189, and Livin β 149-280 express Livin β amino acids 1-189, 80-189, and 149-280 (numbering according to NCBI Reference Sequence NP_071444.1) from pPC97.

Mammalian two-hybrid analyses

The CheckMate Mammalian Two-Hybrid System (Promega, Mannheim, Germany) was employed to analyze the Livin/peptide and Livin/Smac interactions in mammalian cells. Full-length Livin α and β proteins, as well as individual Livin β deletion mutants (Livin β 1-189, Livin β 80-189, and Livin β 149-280), were expressed as GAL4BD fusion proteins from pBIND [27]. Coding sequences for individual Livin-binding peptides were fused to VP16-AD in pACT (pACT-Lp). pACT-Smac has been described previously [27]. Both pBIND and pACT fusion constructs were transfected into HeLa cells, together with the GAL4-responsive luciferase reporter construct pG5luc and the internal standard pCMV-Gal. Cells were harvested 24 h after transfection, luciferase activities were determined as duplicates in at least three independent experiments, and normalized for pCMV-Gal activities, as described [28].

Treatment with pro-apoptotic stimuli and TUNEL analyses

For treatment with pro-apoptotic stimuli, cells grown on cover slips were exposed 32 h post-transfection to a single dose of 50 J/m^2 UV-irradiation (Stratalinker 2400, Stratagene, Heidelberg, Germany) or to 50 µg/ml etoposide (Sigma, Taufkirchen, Germany), and harvested 16 h later. Terminal deoxynucleotidyltransferase-mediated UTP end-labeling (TUNEL) analyses were performed for detection of apoptosis, using the in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics). Apoptotic strand breaks and total DNA were visualized by a Vanox-T AH-2 epifluorescence microscope (Olympus, Center Valley, PA, USA). Pictures were captured with an F-View camera (Soft Imaging System, Olympus) and analyzed with analySIS^B software (Soft Imaging System).

Protein analyses

Cellular proteins were extracted in RIPA buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). For Western-blot analyses, 30 µg of protein were separated by 12.5% SDS–PAGE, transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA), and analyzed by enhanced chemiluminescence (GE Healthcare, Munich, Germany). The following antibodies were employed: monoclonal anti-Livin antibody (Active Motif, Carlsbad, CA, USA), monoclonal anti-Livin antibody no. 6 [9], monoclonal anti-α-Tubulin antibody (Calbiochem, Darmstadt, Germany), and polyclonal rabbit anti-caspase-3 antibody Pab CM1 (BD Biosciences, Heidelberg, Germany).

For immunofluorescence analyses, cells were grown on cover slips and fixed in 4% paraformaldehyde. Livin protein was detected by monoclonal anti-Livin antibody no. 6 or, as HA-tagged protein, with anti-HA mouse monoclonal antibody (Sigma), and expression of H6-tagged Livin-binding peptides by monoclonal anti-His antibody [29]. Polyclonal anti-mouse IgG-Cy3 (Dianova, Hamburg, Germany) was used as secondary antibody. Nuclei were stained with DAPI. Results were visualized by epifluorescence microscopy.

Colony formation assays

Cell lines were transfected with individual pIRESneo-H6R9-peptide expression vectors, as indicated. Transfected cells were selected by neomycin resistance with Geniticin (Invitrogen). After 10–14 days selection, colonies were fixed with formaldehyde and stained with crystal violet.

Statistical analyses

Statistical analyses were performed applying the two-tailed paired Student's *t*-test. The GraphPad QuickCals software (GraphPad Software, Inc., La Jolla, CA, USA) was used. $p < 0.05$ was considered to be statistically significant.

Results

Isolation of peptides binding to Livin β

A yeast two-hybrid (Y2H) interaction screen was performed in order to identify linear peptides that bind to Livin. Since the Livin α isoform exhibited intrinsic transactivation activity in yeast, Livin β was used as bait. As prey, we employed an expression-library encoding randomized linear 20-mer peptides (flanked by glycine–proline on both termini) linked to the transcriptional activation domain of GAL4 [22]. Screening was performed using yeast strain KF1 [21]. From 4×10^6 yeast transformants, we isolated 90 clones that activated both the *ADE2* and the *HIS3* selection markers of KF1 in replica analyses. Among these, 46 also stimulated the *URA3* marker of KF1, which is only activated by relatively strong protein–protein interactions [21].

Sequence comparison of the deduced peptide sequences showed that all 46 Livin-binding peptides were longer than the expected 24 amino acids, due to a frame-shift which was present in less than 30% of the peptides in the library. This resulted in additional, vector-borne amino acids at the C-terminus of the Livin β-targeting peptides. Data bank analyses indicated that all peptides did not show obvious sequence homologies to known Livin interaction partners, such as Smac, or to other human proteins.

In order to assess the specificity of Livin β/peptide interactions, additional yeast two-hybrid analyses were performed with a series of control proteins. None of the Livin-binding peptides interacted with unrelated proteins, such as HPV16 E6 protein, or with more closely related other members of the IAP family, such as c-IAP-1, c-IAP-2, XIAP, and Survivin (Table 1), demonstrating high binding specificity for Livin β.

Peptide binding to Livin α and β in mammalian cells

In order to investigate whether the peptides bind to Livin in mammalian cells as well, the CheckMate assay was employed. In this mammalian two-hybrid system, the intracellular interaction of two binding partners leads to the transcriptional activation of a co-transfected luciferase reporter gene [28]. We observed that approximately 50% (22 from 46) of the peptides also detectably interacted with

Table 1 Sequences and binding specificities in yeast of Livin-binding peptides

Peptide	Amino acid sequence	Livin β	c-IAP-1	c-IAP-2	XIAP	Survivin	HPV16 E6
Lp15	GSGCGCFVRGRIVRIRCVILLLRVLRS*	+	–	–	–	–	–
Lp16	GSGLCVRRWWGMSVGSRIMLVMLVLRS*	+	–	–	–	–	–
Lp40	GSGCVRIRVGIVRRMLFLRFVFLVLRS*	+	–	–	–	–	–
Lp65	GAGLGRVIRLRIVVLRCIFLLFRVLRS*	+	–	–	–	–	–
Lp75	GSRCIRRRISILFFVFRVLRSRRVLRS*	+	–	–	–	–	–
Lp90	GPYPYLRILLVQKIACVRRALWVLRS*	+	–	–	–	–	–
K22	GPFDDSNGPCFYCWDNSCKGRGGP*	–	–	–	–	–	–

Binding studies were performed in yeast strain KF1. (+) binding in yeast two hybrid assay, (–) no detectable interaction

Lp Livin-binding peptides (sequences include their common vector-borne VLRS motive at the C-terminus), *K22* Control peptide

Livin β in mammalian cells (data not shown). For further analyses, we chose six peptides that exhibited the strongest binding activities in yeast two-hybrid and in CheckMate assays. A control peptide K22 that did not exhibit unspecific toxic effects was chosen arbitrarily from the peptide expression library. The amino acid sequences of the peptides and their binding properties in yeast are summarized in Table 1.

In contrast to the situation in yeast, Livin α did not exert detectable intrinsic transactivation activity in mammalian cells. This allowed us to perform CheckMate-binding assays with both Livin isoforms. All selected peptides clearly interacted with Livin α and Livin β (Fig. 1). Moreover, binding of the peptides to the two Livin isoforms was highly specific in mammalian cells, in that no interaction with control proteins, including closely related IAPs (c-IAP-1, c-IAP-2, XIAP), was detectable (data not shown).

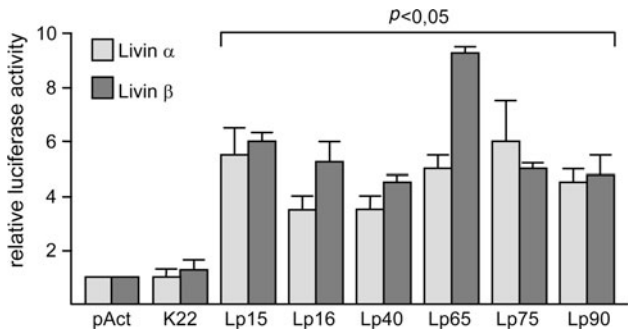


Fig. 1 Peptides binding to Livin α and Livin β in mammalian cells. CheckMate analyses upon cotransfection of HeLa cells with pBIND-Livin α or pBIND-Livin β, respectively, together with pACT vectors encoding the indicated Livin-binding peptides (Lp). Luciferase values are indicated relative to the activities of pBIND-Livin α or pBIND-Livin β, respectively, in the presence of cotransfected control vector pACT (arbitrarily set at 1.0). *K22* negative control. Standard deviations and *p*-values are indicated

All peptides bind to the central, BIR domain-containing region of Livin

Livin contains two important functional domains: the BIR (baculovirus IAP repeat) domain, which is involved in interactions with caspases and Smac, and the RING (really interesting new gene) finger domain, which exerts E3 ubiquitin ligase activity [30]. To delineate the Livin domains bound by the peptides, we created Livin β deletion mutants. Livin β 1-189 and Livin β 80-189 both contain the BIR domain (amino acids 83-167). Livin β 149-280 includes the RING finger (amino acids 228-280) plus spacer sequences, but lacks the BIR domain [8]. In Y2H analyses all peptides bound to Livin β 1-189, but not to Livin β 149-280 (Fig. 2a). Thus, the RING finger domain was not required for the interaction of the peptides with Livin. In addition, Livin sequences N-terminal to the BIR domain were not required for binding, since all peptides also bound to Livin β mutant 80-189. The binding pattern of the peptides in yeast (Fig. 2a) corresponded to their binding pattern in mammalian cells (Fig. 2b). These findings identify the BIR domain-containing amino acids 80-189 as sufficient for binding by all tested Livin-binding peptides.

Intracellular expression of the Livin-binding peptides sensitizes *livin*-expressing tumor cells toward pro-apoptotic stimuli

Next, we assessed whether the Livin-binding peptides can sensitize cancer cells toward apoptosis. In this experimental setting, individual peptides were expressed in Livin-positive (HeLa, Caki-1, and MeWo), and in Livin-negative (H1299) cells, as previously defined [5, 10]. Intracellular expression of all peptides was verified by immunofluorescence microscopy, using an antibody against their His-Tag (data not shown).

Cells were treated with the pro-apoptotic agents UV-irradiation or etoposide, and apoptotic cells were visualized by TUNEL analyses. We found that all Livin-binding

a

Peptide	Livin β 1-189	Livin β 80-189	Livin β 149-280
Lp15	++	+	-
Lp16	++	+	-/(+)
Lp40	+	+	-
Lp65	+	+	-
Lp75	++	+	-
Lp90	++	+	-
K22	-	-	-

b

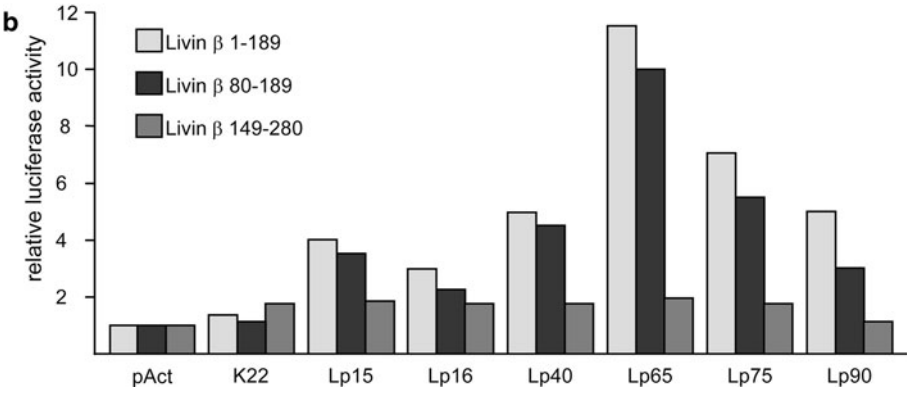


Fig. 2 Livin-binding peptides interact with the central, BIR domain-containing region of Livin. **a** Binding studies in yeast strain KF1. Livin β deletion mutants were expressed from pPC97. (++) activation of two marker genes (*GAL2-ADE2* and *GAL1-HIS3*), (+) activation of one marker gene (*GAL1-HIS3*), (−) no activation. *Lp* Livin-binding peptide, *K22* control peptide. **b** CheckMate assays in HeLa cells with Livin β deletion mutants expressed from pBIND and individual peptides expressed from pACT. Activation of the luciferase reporter relative to cotransfection with control vector pACT (arbitrarily set at 1.0)

peptides significantly increased the apoptosis rate in HeLa cells, following treatment with either pro-apoptotic agent (Fig. 3a, b). In accordance with the TUNEL data, we observed that expression of Livin-targeting peptides, but not of a control peptide, led to an activation of effector caspase-3 in HeLa cells (Fig. 3c). Likewise, pro-apoptotic sensitization was observed upon expression of the Livin-binding peptides in Livin-positive Caki-1 and MeWo cells (Fig. 3b). On the contrary, all Livin-binding peptides did not exert pro-apoptotic activities in Livin-negative H1299 cells (Fig. 3a, b) above that of K22-expressing control transfectants. Thus, the Livin-targeting peptides sensitized *livin*-expressing cells toward pro-apoptotic stimuli.

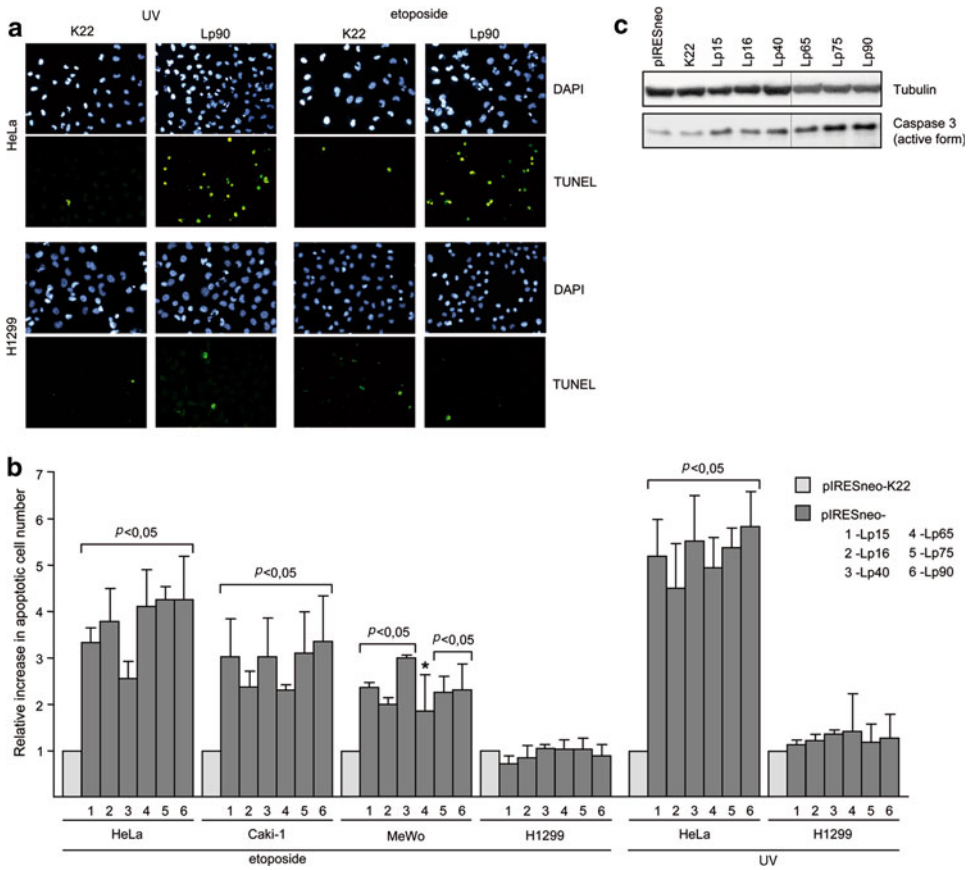
Livin-binding peptides do not alter the level or intracellular localization of Livin

It was reported that small molecule IAP antagonists can induce autoubiquitination and rapid proteasomal degradation of c-IAPs as a possible inhibitory mechanism [31, 32]. In case that Livin-inhibiting peptides also stimulated autoubiquitination and subsequent proteasomal degradation, a reduction of intracellular Livin amounts would be expected. Expression of Livin-binding peptides, however, did not affect the concentrations of endogenous Livin in HeLa cells (Fig. 4a). Likewise, they did not alter the levels of ectopically co-expressed Livin β in H1299 cells (Fig. 4b).

Peptide aptamers can inhibit certain target proteins by sequestration into aggresomes [33]. In order to determine whether the Livin-targeting peptides may act through a similar mechanism, we analyzed the effects of Livin-binding peptides on the intracellular localization of Livin, in the presence or absence of pro-apoptotic stimuli. Since endogenous Livin was not detectable by immunofluorescence with available antibodies, we ectopically co-expressed Livin β, together with individual Livin-binding peptides or control peptide K22, in HeLa cells. Livin localized mainly in the cytoplasm, as previously reported [8], but exhibited no obvious differences in its intracellular localization upon co-expression of the bioactive Livin-binding peptides (as shown for Lp90 in Fig. 4c) or control peptide K22. In addition, Livin accumulation within perinuclear inclusion bodies—as indicative for aggresome formation [34]—was not observed. The expression pattern of Livin remained unchanged after treatment with UV-irradiation (Fig. 4c) or etoposide (not shown). Thus, apoptotic sensitization by Livin-binding peptides was not associated with obvious alterations of the intracellular concentration or the subcellular distribution of Livin.

Smac binding to Livin β is not inhibited by Livin-binding peptides

The BIR domain of Livin is bound by Smac, and it has been postulated that this interaction is crucial for the anti-



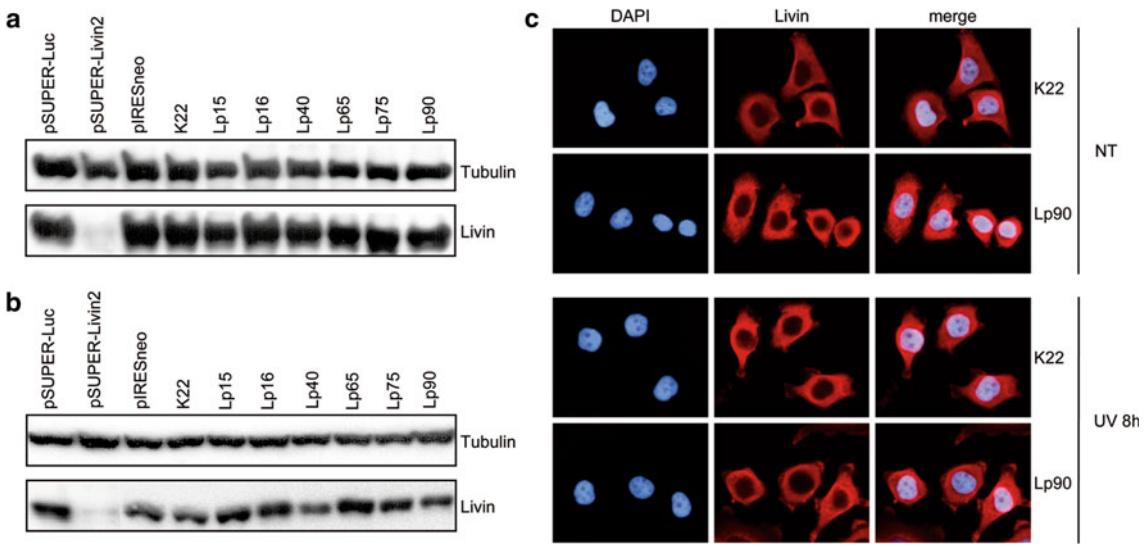


Fig. 3 Expression of Livin-binding peptides sensitizes Livin-expressing tumor cells toward pro-apoptotic stimuli. Livin-positive HeLa, Caki-1, and MeWo, and Livin-negative H1299 cells were transfected with pIRESneo vectors expressing the indicated peptides, and exposed to 50 J/m^2 UV-irradiation or 50 µg/ml etoposide. **a** Detection of apoptotic cells by TUNEL assays. Cell nuclei were visualized by DAPI staining. Magnification: 200-fold. **b** Relative increase in number of TUNEL-positive cells after etoposide or UV treatment. The number of apoptotic cells after transfection of the negative control peptide K22 was set at 1.0. Standard deviations and *p*-values are indicated. *Asterisk* $p = 0.0529$. **c** Western-blot analysis of caspase-3 activation in HeLa cells, after expression of indicated peptides and etoposide treatment

Fig. 4 Livin-binding peptides do not influence expression levels or the intracellular localization of Livin. **a** Western-blot analysis of endogenous Livin α and Livin β protein in HeLa cells after expression of the indicated peptides from pIRESneo. pSUPER-Livin-2, transfection of a shRNA expressing vector blocking expression of both Livin isoforms [10] to validate the immunoblot signals. **b** Western-blot analysis of ectopically expressed Livin β in H1299 cells. Livin β was co-expressed from pcDNA3HA-Livin β, together with the indicated peptides from pIRESneo. **c** Immunoflorescence analysis of the intracellular localization of Livin β. HeLa cells were cotransfected with pcDNA3HA-Livin β, together with pIRESneo expressing Livin-binding peptide (Lp) 90 or control peptide K22, respectively. Cells were either left untreated (*upper panel*) or exposed to 50 J/m^2 UV-irradiation (*lower panel*). *Livin*: detection of HA-Livin β expression by anti-HA antibody. Nuclei were stained with DAPI. Magnification: 400-fold

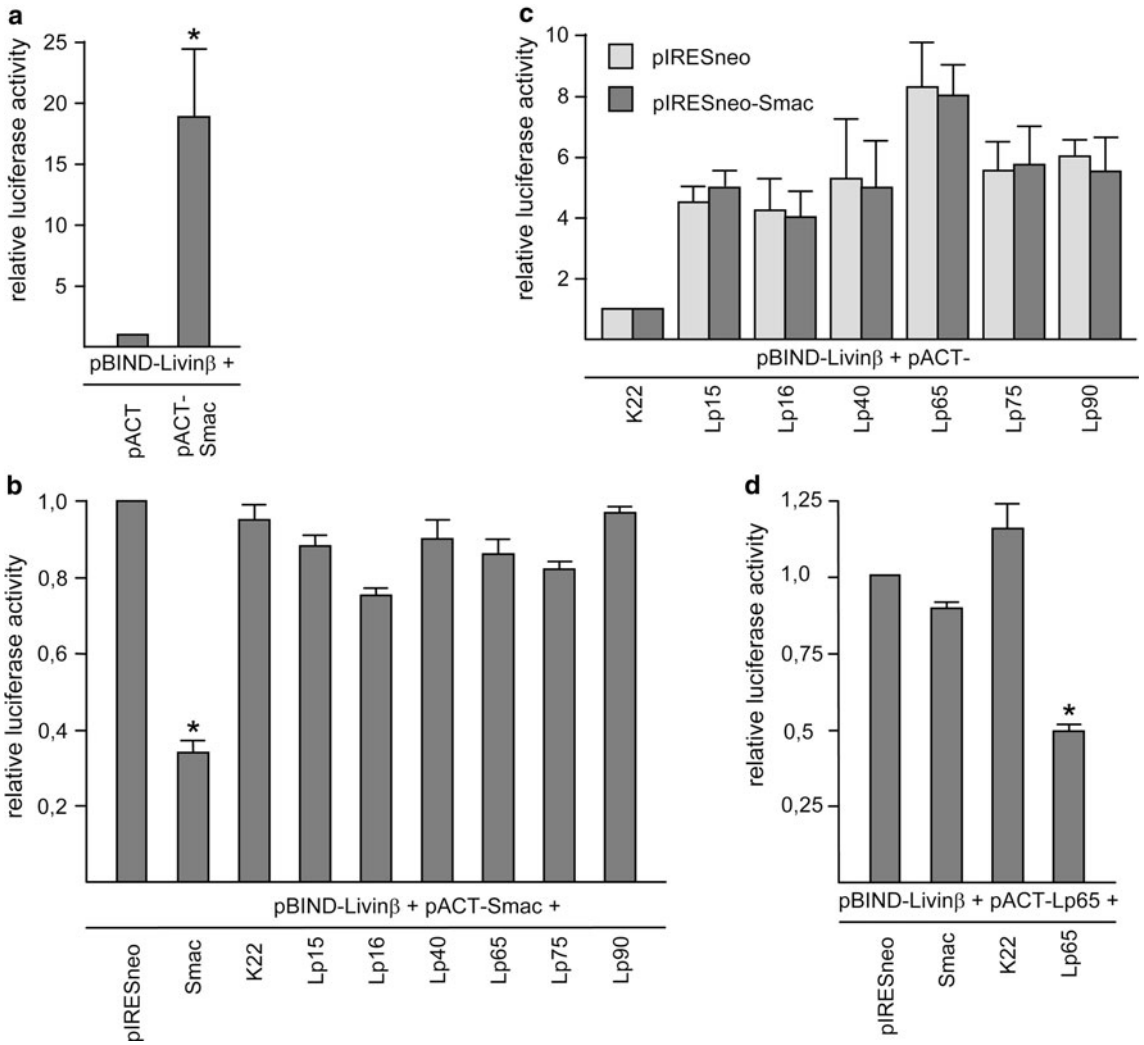


Fig. 5 Livin-binding peptides do not interfere with the binding of Smac to Livin β. CheckMate analyses in HeLa cells. Standard deviations are indicated. *Asterisk* $p < 0.05$. **a** Cotransfection of pBIND-Livin β and pACT or pACT-Smac. Activation of the luciferase reporter is indicated relative to cotransfection with control vector (arbitrarily set at 1.0). **b** Cotransfection of pBIND-Livin β and pACT-Smac, together with pIRESneo-expression vectors coding for Smac, control peptide K22, or individual Livin-binding peptides (Lp). Luciferase activities reflecting the pBIND-Livin β and pACT-Smac interaction (see also **a**) were set at 1.0. **c** Cotransfection of pBIND-Livin β and pACT, expressing control peptide K22 or the indicated Livin-binding peptides (Lp), together with pIRESneo (vector control) or pIRESneo-Smac, respectively. **d** Cotransfection of pBIND-Livin β and pACT-Lp65, together with the indicated pIRESneo-expression vectors for Smac, control peptide K22, or the homologous Lp65 peptide

apoptotic activity of Livin [35]. We therefore investigated whether the Livin-targeting peptides may interfere with the binding of Livin to Smac. The intracellular interaction of Livin β linked to the GAL4-DNA-binding domain and Smac linked to the VP16 transactivation domain is readily detectable in HeLa cells, by mammalian two-hybrid analysis (Fig. 5a). Co-expression of unfused Smac protein substantially reduced luciferase activities (Fig. 5b), as can be expected from its potential to replace Smac-VP16 on Livin β-GAL4. In contrast, however, co-expression of all tested Livin-binding peptides did not affect the Livin β/Smac interaction (Fig. 5b). Vice versa, Smac did not interfere with the interaction of Livin β and individual Livin-binding peptides (Fig. 5c). In contrast to Smac, co-expression of a homologous Livin-binding peptide blocked the corresponding Livin/peptide interaction under the same experimental conditions, as exemplified for Lp65 (Fig. 5d).

Livin-binding peptides interfere with the colony formation capacity of *livin*-expressing cells

Long-term inhibition of Livin expression by RNAi led to a significant inhibition of the colony formation capacity of HeLa cells [10]. In order to analyze the effects of the Livin-binding peptides upon prolonged intracellular expression,

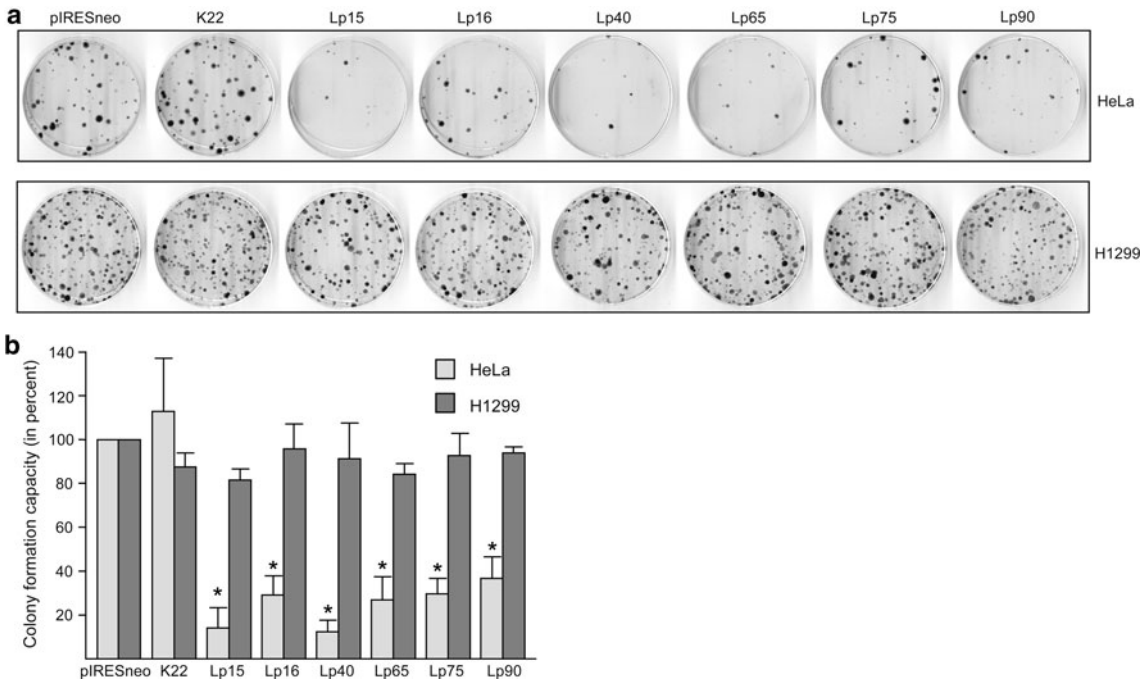


Fig. 6 Livin-binding peptides inhibit the growth of Livin-positive HeLa, but not of Livin-negative H1299 cells, in colony formation assays. **a** Cells were stably transfected with pIRESneo (vector control), or with pIRESneo-vectors expressing control peptide K22 or the individual Livin-binding peptides (Lp), as indicated. After 7–10 days of selection with Geniticin, colonies were fixed with formaldehyde and stained with crystal violet. **b** Quantification of colony formation assays. Colony numbers of control-transfected cells (*pIRESneo*) were set at 100%. Standard deviations are indicated. *Asterisk* $p < 0.05$

we performed colony formation assays following expression of Livin-targeting peptides from stably transfected pIRESneo vectors. We found that spontaneous apoptosis was not considerably increased upon intracellular expression of the Livin-targeting peptides, as assessed by DAPI staining and TUNEL analyses (data not shown). However, all Livin-targeting peptides efficiently blocked the growth of Livin-positive HeLa (Fig. 6) and MeWo (data not shown) cells, compared to cells transfected with the pIRESneo vector alone, or to cells expressing a control peptide. On the contrary, the colony formation capacity of Livin-negative H1299 cells was not affected by any of the tested peptides (Fig. 6). These results show that Livin-targeting peptides have the potential to interfere with the colony formation capacity of *livin*-expressing cells.

Discussion

The anti-apoptotic *livin* gene is preferentially expressed in cancer cells and its targeted inhibition is considered to represent a promising new strategy to overcome the apoptotic resistance of tumor cells [6, 7, 36]. In order to extend the spectrum of potential IAP inhibitors, we here screened a randomized expression library for peptides binding to Livin. A series of linear peptides was identified that bind Livin both in yeast and in mammalian cells with high specificity.

Upon intracellular expression, the peptides efficiently sensibilized Livin-positive cancer cells toward pro-apoptotic stimuli. The spontaneous apoptosis rate (i.e., in the absence of pro-apoptotic stimuli) of Livin-expressing cells was not markedly altered upon intracellular expression of Livin-binding peptides. Similarly, inhibition of Livin-expression by siRNA also did not affect the spontaneous apoptosis rate [10, 37]. Yet, the Livin-targeting peptides strongly inhibited the growth of Livin-positive tumor cells in colony formation assays. This latter finding indicates that the inhibition of Livin—apart from pro-apoptotic sensitization of tumor cells—also exerts anti-proliferative effects. In line with these findings, it has been previously reported that long-term silencing of Livin expression by siRNA blocks colony formation capacity [4, 10] and inhibits cell proliferation [38] of Livin-expressing cells.

The six peptides characterized in the present study share no apparent sequence homologies among each other. In addition, no obvious sequence homologies to naturally occurring Livin-interaction partners, such as Smac or caspases, were observed. This is not unexpected since active, processed Smac requires a free N-terminus with an exposed IAP-binding motif (IBM) for interacting with Livin and other IAPs [35, 39]. A corresponding binding motif

is present also in the exposed N-terminus of the small subunit of processed caspase-9 [40]. For screening purposes, the peptides identified here were fused on their N-terminus to the GAL-AD and therefore cannot expose N-terminal IBM-like sequences.

In contrast to recently described small-molecule SMAC-based IAP antagonists, which lead to increased autoubiquitination and rapid proteosomal degradation of c-IAPs [31, 32], the bioactive peptides isolated in the present study did not decrease intracellular Livin protein levels. Notably, Livin function can be modified through caspase-mediated cleavage and subsequent translocation to perinuclear compartments, thereby transforming Livin from an anti-apoptotic to a pro-apoptotic factor [41–43]. Yet, upon intracellular expression of the Livin-binding peptides, we did not observe an intracellular redistribution of Livin or increased levels of the truncated Livin form (data not shown). We also did not detect a possible sequestration into aggresomes, as observed for some target proteins of inhibitory peptide aptamers [33]. It thus will be interesting for future studies to gain more insights into the detailed mechanism by which the Livin-targeting peptides resensitize toward apoptosis and block cell growth. In this context, it is noteworthy that members of the IAP family have been involved in the regulation of a broad set of different intracellular pathways, which include caspase-independent apoptosis, receptor signalling, cell cycle regulation, proliferation control, morphogenesis, and regulation of immune response [44, 45]. Transcriptome analyses upon intracellular expression of the Livin-targeting peptides in *livin*-expressing cells may be helpful for further deciphering additional downstream pathways which are critical for the pro-apoptotic and anti-proliferative activities of Livin.

Besides being useful for these functional analyses, the pronounced biological effects of the Livin-binding peptides have therapeutic implications. Firstly, the peptides can be valuable tools for the further exploration of Livin as a potential therapeutic target. One main advantage of peptides is that they typically bind to distinct domains of the target protein. They thus could be more predictive for the effects of a therapeutic agent (e.g., of a small molecule inhibitor) than using siRNAs for target evaluation [18]. Secondly, the bioactive Livin-targeting peptides identified here could provide a novel basis for the development of protein therapeutics blocking Livin. However, like protein therapy in general, this endeavor is still complicated by several technical hurdles, including potential immunogenicity, metabolic instability, intracellular protease degradation, and delivery problems of proteinaceous drugs [46, 47]. Thus, the further exploration of Livin-inhibitory peptides as potential therapeutics will require optimization of both biopharmaceutical and pharmacokinetic parameters. In this context, it is noteworthy that relatively simple alterations of IAP-binding peptides, such as the addition of a single methyl group, can lead to significant improvements in both affinity and selectivity [48]. Thirdly, it could be envisioned to employ the Livin-inhibiting peptides as a basis for design of peptidomimetics. This approach has been successfully followed by the development of Smac mimetics blocking IAPs [17, 19]. Fourthly, it is likely that the Livin-binding peptides, which induce therapeutically desired cellular phenotypes such as pro-apoptic sensitization, bind to functionally important domains on Livin. Conceivably, small molecules binding to the same or to an overlapping region could act as functional peptide mimics which should form a valuable basis for screening compound libraries by displacement assays [18]. Such small molecules can be preselected for their drug-likeness, structural and shape diversity, and compliance with medicinal chemistry requirements, thereby circumventing many of the pharmacological problems still associated with protein therapeutics. In conclusion, the peptidic Livin inhibitors isolated in this study should be useful both for structural and functional analyses of Livin in basic research as well as for the development of novel therapeutic strategies blocking Livin in tumor cells.

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