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Cloning and characterization of the rat homologues of the Inhibitor of Apoptosis protein 1, 2, and 3 genes.

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Abstract

Background: Inhibitor of Apoptosis (IAP) proteins are key intrinsic regulators of apoptosis induced by a variety of triggers. We isolated the rat Inhibitor of Apoptosis genes 1, 2 and 3 and characterized their tissue distribution and expression.

Results: Rat *iap-1* encodes a protein of 67.1 kDa with 73 % and 89.2 % homology to human and mouse *iap-1* respectively. Rat *iap-2* encodes a protein of 66.7 kDa with 81.6 % and 89.3 % homology to human and mouse *iap-2* respectively. Rat *iap-3* encodes a protein of 56.1 kDa with 89.5 % and 93.1 % homology to human and mouse *iap-3* respectively. We have generated rabbit polyclonal antibodies against all three rat IAP genes. Northern and Western blot analysis detected rat IAP transcripts and proteins in majority of the tissues examined. In addition, a shorter, alternatively spliced transcript corresponding to *iap-2* was found in testes.

Conclusions: We have identified three rat homologues of the IAP genes. The elevated expression of rat *iap-1* and *iap2* in testes suggests that these two genes play an important antiapoptotic role in spermatogenesis.

Background

Apoptosis, or programmed cell death, is a naturally occurring process that is required for normal development in multicellular organisms as well as for defense against viral infections and the emergence of cancer [1]. The Inhibitor of Apoptosis proteins (IAP) are a family of novel genes that function in the cell death pathway to block apoptosis induced by a variety of triggers [reviewed in [2–4]]. It was shown recently that the mechanism by which the IAPs inhibit apoptosis is direct inhibition of key apoptotic proteases, caspase 3 and 7 [5–7]. The IAPs were initially discovered in baculoviruses, but their homologues have since been identified in other viruses, mammals, birds and insects, suggesting a common evolutionary origin [reviewed in [2,3,8]]. There is a growing body of published reports investigating the role of the IAP genes *in vivo* using rat as a model system (e.g. [9–11]). However, although partial and complete nucleotide sequences of the rat IAP homologues were recently submitted to GenBank by several groups the rat IAP genes and their expression were not

characterized. To facilitate future studies in the rat models we report here the isolation and characterization of rat cDNAs homologous to *hiap-1*, *hiap-2* and *xiap* as well as the generation of specific anti-IAP antibodies. We also examine the tissue distribution of the rat IAPs both on the mRNA and the protein levels.

Results and Discussion

The rat homologues of IAPs were isolated from rat brain cDNA library as described in Materials and Methods. The rat IAPs are similar in the sequence composition to both the human and mouse IAPs and to the rat sequences available in the public GenBank database (AF190020, AF081503, and AF033366). The general structure of all three rat IAPs (three BIR domains, linker region and a RING zinc finger) is consistent with the human and mouse proteins indicating functional conservation of these proteins (not shown). The rat *iap-1* (AF183430) open reading frame encodes a 603 amino acid protein with a predicted molecular weight of 67.1 kDa and shows 76.8 % (DNA) and 73 % (protein) homology to *hiap-1*[12], and 90.7 % (DNA) and 89.2 % (protein) homology to murine *miap-1*[13]. The rat *iap-2* (AF183431) open reading frame encodes a 590 amino acid protein with a predicted molecular weight of 66.7 kDa. The rat *iap-2* shows 82.8 % (DNA) and 81.6 % (protein) homology to *hiap-2*[12], and 91.2 % (DNA) and 89.3 % (protein) homology to murine *miap-2*[13]. The rat *iap-3* (AF183429) open reading frame encodes a 496 amino acid protein with a predicted molecular weight of 56.1 kDa. The rat *iap-3* shows 89.4 % (DNA) and 89.5 % (protein) homology to x*iap*[12], and 93.8 % (DNA) and 93.1 % (protein) homology to murine *miap-3*[14].

Northern blot analysis of multiple tissue poly(A)$^+$ blot (Clontech) using a coding region of each rat *iap* as a probe revealed tissue distribution similar to that observed for human or mouse IAPs. The rat *iap-1* transcript is approximately 3.5 kb and is most abundantly expressed in testes, followed by spleen and liver (Figure 1). We did not detect any *iap-1* transcript in brain, lung, skeletal muscle or kidney. In contrast, *iap-2* transcript is approximately 4.3 kb and is expressed in all tissues examined with the highest expression in testes, followed by liver and heart (Figure 1). We also observed the presence of higher molecular weight bands for both the *iap*-1 and *iap*-2 similar to those observed for mouse *iap*[13]. The nature of these bands is not known at present but they likely represent transcripts generated by a polyadenylation signal readthrough. It has been demonstrated at least for the human IAPs that they contain several alternative polyadenylation sites in their respective 3' untranslated regions (UTRs) [15]. The rat *iap-3* transcript is approximately 8 kb in size. The expression of rat *iap-3* is highest in liver, followed by heart and spleen (Figure 1). We did not detect any *iap-3* expression in kidney and testes.

It is interesting to note the presence of an additional, shorter *iap-2* transcripts in testes (approximately 2.0 and 3.5 kb) that are not present in other tissues. The 3.5 kb transcript is likely an *iap*-1 cross-hybridizing band which is also present strongly in testes (Figure 1, top). The shorter transcript, however, represents an alternative, testis-specific splice variant of *iap-2*. This hypothesis is supported by the fact that the same transcripts are detected on Northern blots hybridized with different fragments of *iap-2* coding region (Figure 1B). These data suggest that the testis-specific splice variants of *iap-2* contain both the BIR and the RING zing finger regions. Thus the alternative splicing has likely occurred within the 5' or 3' UTR regions. Another possibility is that the shorter transcript could represent a novel, testis-specific *iap* gene which cross-hybridizes to the *iap-2* probe. In fact, a shorter, testis-specific *xiap*-like transcript termed TIAP or ILP-2 has been recently isolated from human testes [16,17]. It has been shown that TIAP/ILP-2 is an intronless gene homologous to the C-terminal end of XIAP. However, and unlike XIAP, TIAP/ILP-2 was able to protect only against BAX-pathway induced apoptosis.

We next generated rabbit polyclonal antibodies against bacterially expressed and purified GST fusion proteins of each of the three rat IAPs and used these antibodies to examine the rat IAP distribution in multiple tissues (Figure 2). The protein samples were prepared from fresh adult rat tissues and analyzed by Western blot. The rat IAP-1 was expressed in all tissues examined with the exception of brain, liver and thymus. The expression of IAP-2 was observed in all tissues except brain, lung and thymus. In contrast, IAP-3 was found in all tissues examined. In the majority of the tissues we observed one prominent protein band of the expected molecular mass for each of the rat IAPs. Several tissues (intestine, kidney, spleen, testes), however, showed smaller protein bands of varying intensity which could be detected with the anti-*iap-1* and anti-*iap-2* antibodies, but not the anti-*iap-3* antibody. The corresponding shorter mRNA transcripts that could give rise to smaller protein bands were only observed in testes and only for *iap-2* mRNA. The nature of the smaller proteins and their function compels future investigations. It is possible that due to the high sequence homology our polyclonal antibodies to IAP-1 and IAP-2 cross-react with both proteins. However, this seems unlikely since the anti-*iap-1* antibody detects a protein band in lung that is not recognized by the anti-*iap-2* antibody. Similarly, a protein band in the liver sample is recognized by the anti-*iap-2* antibody, but not the anti-*iap-1* antibody. The expression profile detected by Western analysis seems to contradict that of the Northern blots (Table 1). It is likely, however,

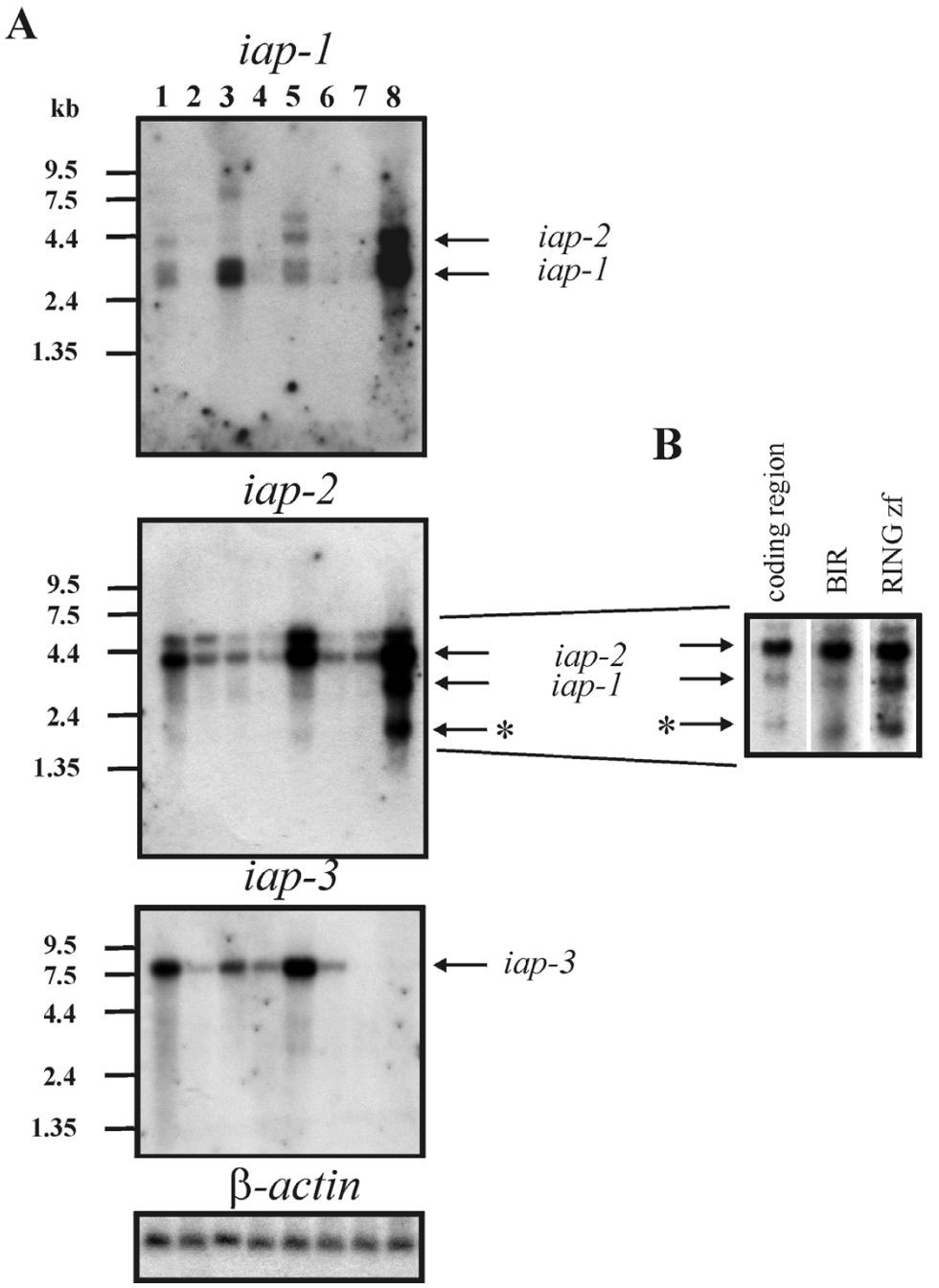

Figure 1

(**A**) Northern blot analysis of rat *iap* mRNA expression in adult rat tissues. A rat multiple tissue northern blot (Clontech) containing 2 μg/lane poly(A)$^+$ RNA *per* lane was probed sequentially with [^{32}P] dCTP (Amersham) labeled, random primed (Amersham Rediprime) DNA probes derived from the coding regions of rat *iap-1*, *iap*-2, *iap-3*, and β-actin (control). Blots were hybridized overnight in 5 × SSPE/10 × Denhardt's solution/100 μg/ml salmon sperm DNA/ 50% formamide/ 2% SDS and then washed with 0.2 × SSC/ 0.1% SDS at 50°C. The position and sizes of the markers are indicated on the left. The tissues represented on the blot are as follows: (**1**) heart, (**2**) brain, (**3**) spleen, (**4**) lung, (**5**) liver, (**6**) skeletal muscle, (**7**) kidney, and (**8**) testis. (**B**) Northern blot analysis of the expression of *iap*-2 in testes. A rat multiple tissue Northern blot (Clontech; same as in (A)) was hybridized sequentially with [^{32}P] dCTP labeled DNA probes specific to *iap*-2 coding region, BIR domain and the RING zing finger. The blots were processed as in (A). The position of testis-specific transcript is indicated by an asterisk (*).

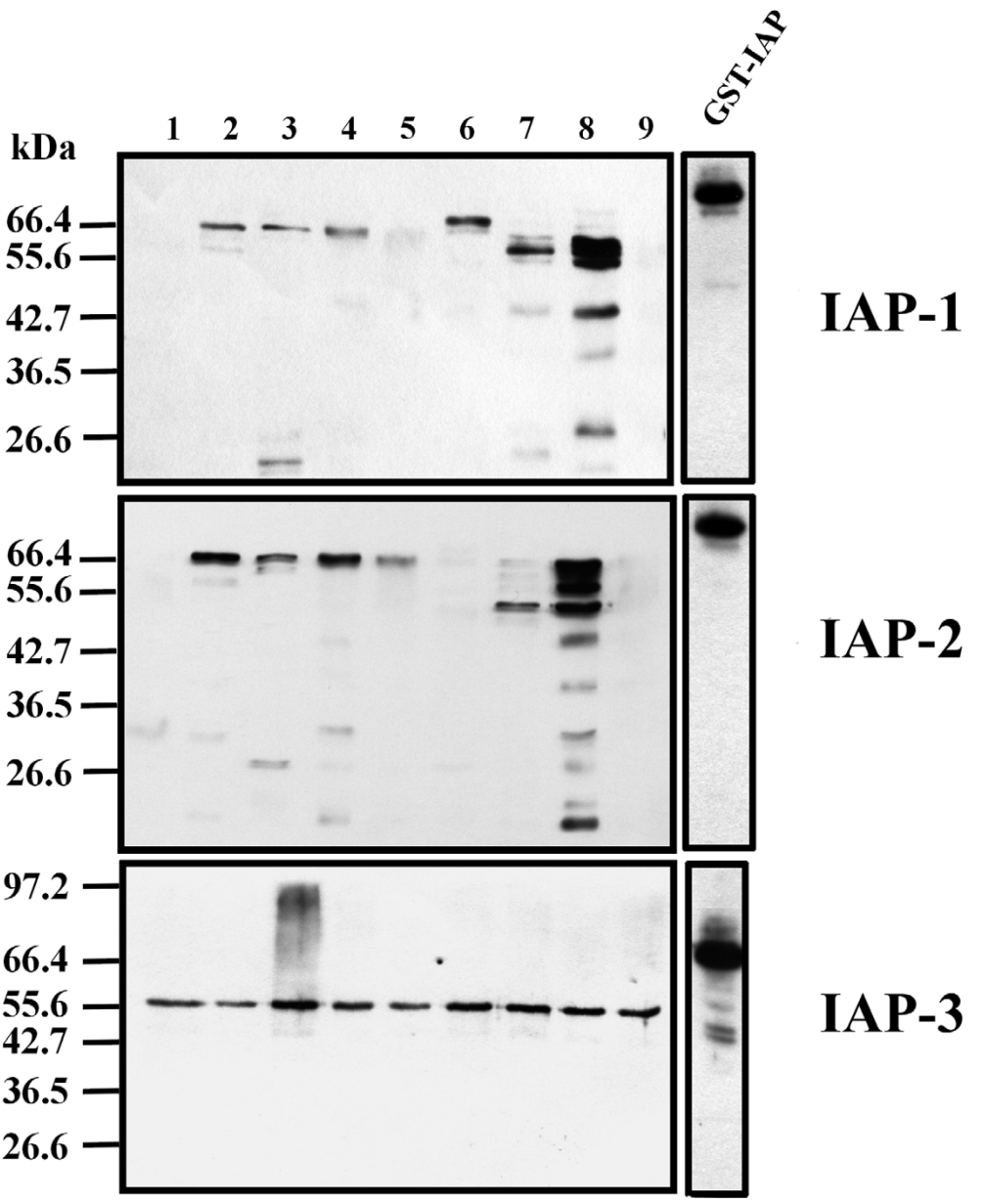

Figure 2
Western blot analysis of the rat IAP proteins distribution in adult rat tissues. The protein samples prepared from fresh adult rat tissues, or bacterially expressed GST-fusion recombinant proteins, were separated on 10% SDS-PAGE (12 μg total protein *per* lane) and transferred onto a PVDF membrane using standard techniques [20]. Rabbit polyclonal antibodies against rat *iap-1* (top), *iap-2* (middle), and *iap-3* (bottom) were used at 1:1,000 dilution followed by secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Amersham) used at 1:1,500 dilution. Antibody complexes were detected using the ECL system (Amersham). Position and size of the markers is indicated on the left. (Note that the recombinant GST-fusion proteins are larger due to the presence of N-terminal GST tag.) Tissues used are as follows: (**1**) brain, (**2**) heart, (**3**) intestine, (**4**) kidney, (**5**) liver, (**6**) lung, (**7**) spleen, (**8**) testis, and (**9**) thymus.

Table 1: The relative expression levels of the rat *iap* genes in various tissues as detected by Northern or Western blot analysis.

	IAP-1		IAP-2		IAP-3	
	Northern	Western	Northern	Western	Northern	Western
Heart	+	++	++	++*	++	++
Brain	-	-	+	-	+	++
Spleen	++	++*	+	++*	++	++
Lung	-	++	+	-	+	++
Liver	+	+	++	++	++	++
Sk. muscle	-	N/A	+	N/A	+	N/A
Kidney	-	++*	+	++*	-	++
Testis	++	++*	++*	++*	-	++
Intestine	N/A	++*	N/A	++*	N/A	++
Thymus	N/A	-	N/A	-	N/A	++

*indicates the presence of an alternate band (- not expressed; + expressed; ++ highly expressed; N/A - not tested)

that the mRNA levels are very low in some tissues (such as lung and kidney) not allowing for detection by Northern blot. In addition, all three *iap* genes are characterized by unusually long 5' and 3' untranslated regions that could affect translation of respective proteins [2]. Indeed, translational control *via* an Internal Ribosome Entry Site (IRES) has been demonstrated for the expression of XIAP [18].

Conclusions

We have described here the isolation and characterization of rat homologues of *iap*-1, 2 and 3 genes. Furthermore, we have generated rabbit polyclonal antibodies and characterized the expression pattern of rat *iap* 1, 2, and 3 genes in multiple tissues by Northern and Western blot analysis. The rat IAPs are similar in their structure and tissue distribution to their human and mouse homologues. All three genes are expressed in most tissues examined with the notable exception of brain, in which rat *iap-1* and *iap-2* were not detected. In testes we observed additional, shorter mRNA transcript and smaller protein bands of rat *iap-2*. While the function(s) of this testis-specific protein(s) is unknown at present the fact that both *iap-1* and *iap-2* genes are expressed very strongly in testes suggests an important antiapoptotic role of these genes in spermatogenesis. In other tissues, including intestine, kidney and spleen, we detected shorter protein bands as well even though we did not detect corresponding mRNA transcripts. The nature and the function of these proteins is unknown at the present time.

Materials and Methods

Isolation of rat IAPs

A λgt11 rat brain cDNA library (Clontech) was screened by hybridization with mouse *miap-1*, *miap-2* or *miap-3* coding region cDNA probes [13,14] Multiple phage isolates were arranged in a contiguous array by restriction analysis and sequenced using a model 373 ABI automated sequencer. Sequencing of the cDNA clones revealed that only rat *iap-2* and *iap-3* were isolated. Since we were not successful in the isolation of the rat *iap-1* cDNA from the brain cDNA library we cloned it by RT-PCR using total RNA isolated from an adult rat brain tissue and a high fidelity DNA polymerase (*Pfu*, Pharmacia). The oligonucleotide primers used for RT-PCR were derived from mouse *iap-1* sequences (5'-dCCATGAACATGGTTCAAGACAGCGCC and 5'-dCAGGAGAGAAATGTGCGCATCGTGCCC ; [13] encompassing the entire coding region. Multiple RT-PCR products were cloned into pCR2.1 TOPO vector (Invitrogen) and sequenced.

Northern blot analysis

A rat multiple tissue northern blot (Clontech) containing 2 μg/lane poly(A)$^+$ RNA *per* lane was probed sequentially with [^{32}P] dCTP (Amersham) labeled, random primed (Amersham Rediprime) DNA probes derived from the coding regions of rat *iap-1*, *iap-2*, and *iap-3*, or β-actin, respectively . Blots were hybridized overnight in hybridization buffer (5 × SSPE/ 10 × Denhardt's solution/100 μg/ml salmon sperm DNA/ 50% formamide/ 2% SDS) and then washed with 0.2 × SSC/ 0.1% SDS at 50°C.

Generation of antibodies and Western blot analysis.
Rabbit polyclonal antibodies were generated against bacterialy expressed and purified GST fusion proteins of each of the three rat IAPs. The protein samples for Western blot analysis were prepared from fresh adult rat tissues as described [19], separated on 10% SDS-PAGE (12 µg total protein *per* lane) and transferred onto a PVDF membrane using standard techniques [20]. Rabbit polyclonal antibodies against rat *iap-1* (top), *iap-2* (middle), and *iap-3* (bottom) were used at 1:1,000 dilution followed by secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Amersham) used at 1:1,500 dilution. Antibody complexes were detected using the ECL system (Amersham).

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References

1. White E: **Life, death, and the pursuit of apoptosis.** *Genes Dev.* 1996, **10**:1-15
2. Liston P, Young SS, Mackenzie AE, Korneluk RG: **Life and death decisions: the role of the IAPs in modulating programmed cell death.** *Apoptosis* 1997, **2**:423-441
3. Deveraux QL, Reed JC: **IAP family proteins-suppressors of apoptosis.** *Genes Dev* 1999, **13**:239-252
4. Holcik M, Korneluk RG: **XIAP, the guardian angel.** *Nat Rev Mol Cell Biol* 2001, **2**:550-556
5. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC: **The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases.** *Embo J* 1997, **16**:6914-6925
6. Deveraux QL, Takahashi R, Salvesen GS, Reed JC: **X-linked IAP is a direct inhibitor of cell-death proteases.** *Nature* 1997, **388**:300-304
7. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC: **IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases.** *Embo J* 1998, **17**:2215-2223
8. LaCasse EC, Baird S, Korneluk RG, MacKenzie AE: **The inhibitors of apoptosis (IAPs) and their emerging role in cancer.** *Oncogene* 1998, **17**:3247-3259
9. Korhonen L, Belluardo N, Lindholm D: **Regulation of X-Chromosome-Linked Inhibitor of Apoptosis Protein in Kainic Acid-Induced Neuronal Death in the Rat Hippocampus.** *Mol Cell Neurosci* 2001, **17**:364-372
10. Li J, Kim JM, Liston P, Li M, Miyazaki T, Mackenzie AE, Korneluk RG, Tsang BK: **Expression of inhibitor of apoptosis proteins (IAPs) in rat granulosa cells during ovarian follicular development and atresia.** *Endocrinology* 1998, **139**:1321-1328
11. Xu D, Bureau Y, McIntyre DC, Nicholson DW, Liston P, Zhu Y, Fong WG, Crocker SJ, Korneluk RG, Robertson GS: **Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus.** *J Neurosci* 1999, **19**:5026-5033
12. Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, Farahani R, McLean M, Ikeda JE, MacKenzie A, Korneluk RG: **Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes.** *Nature* 1996, **379**:349-353
13. Liston P, Lefebvre C, Fong WG, Xuan JY, Korneluk RG: **Genomic characterization of the mouse inhibitor of apoptosis protein-1 and 2 genes.** *Genomics* 1997, **46**:495-503
14. Farahani R, Fong WG, Korneluk RG, MacKenzie AE: **Genomic organization and primary characterization of *miap-3*: the murine homologue of human X-linked IAP.** *Genomics* 1997, **42**:514-518
15. Young SS, Liston P, Xuan JY, McRoberts C, Lefebvre CA, Korneluk RG: **Genomic organization and physical map of the human inhibitors of apoptosis: HIAP1 and HIAP2.** *Mamm Genome* 1999, **10**:44-48
16. Lagace M, Xuan JY, Young SS, McRoberts C, Maier J, Rajcan-Separovic E, Korneluk RG: **Genomic organization of the x-linked inhibitor of apoptosis and identification of a novel testis-specific transcript.** *Genomics* 2001, **77**:181-188
17. Richter BW, Mir SS, Eiben LJ, Lewis J, Reffey SB, Frattini A, Tian L, Frank S, Youle RJ, Nelson DL, Notarangelo LD, Vezzoni P, Fearnhead HO, Duckett CS: **Molecular cloning of ilp-2, a novel member of the inhibitor of apoptosis protein family.** *Mol Cell Biol* 2001, **21**:4292-4301
18. Holcik M, Lefebvre CA, Yeh C, Chow T, Korneluk RG: **A new internal-ribosome-entry-site motif potentiates XIAP-mediated cytoprotection.** *Nature Cell Biology* 1999, **1**:190-192
19. Conte D, Liston P, Wong JW, Wright KE, Korneluk RG: **Thymocyte-targeted overexpression of xiap transgene disrupts T lymphoid apoptosis and maturation.** *Proc Natl Acad Sci U S A* 2001, **17**:17
20. Harlow E, Lane D: *Antibodies. A laboratory manual.: Cold Spring Harbor Laboratory;* 1988