MATERIALS AND METHODS

Isolation of midbodies

Midbody purification was adapted from techniques developed by Mullins and McIntosh (1982), Sellitto and Kuriyama (1992), and Kuriyama and Ensrud (1999). CHO cells synchronized by successive thymidine and nocodazole treatments were isolated in a taxol and phalloidin-containing medium after furrow ingression to stabilize the midbody structure. Following lysis in a hypotonic buffer that included Triton X-100, insoluble midbodies were pelleted at 2000xg in 40% glycerol.

Characterization of midbody proteins using multidimensional protein identification technology (MudPIT)

Precipitated midbody protein preparations were dissolved in digestion buffer, digested by trypsin, and analyzed by LC/LC/MS/MS according to published protocols (4). Approximately 100 µg of protein was used for a 12-step LC/LC/MS/MS experiment and a total of four experiments were performed. MS/MS spectra obtained were analyzed by SEQUEST using a non-redundant mammalian database. The SEQUEST outputs were then analyzed by DTASelect (5). The DTASelect filter settings were: XCorr: +1 ions 1.8, +2 ions 2.5, +3 ions 3.8; delta CN: 0.08; only half or full tryptic peptides were considered; all subset proteins were removed (the “-o” option in DTASelect). Proteins with 4-5 peptides that passed the DTASelect filter were considered real hits. Proteins with one to four peptides that passed the DTASelect filter were further manually validated.

Bioinformatics & determination of homologues & paralogs

Systematic elimination of 417/577 proteins was performed manually using data from Proteome, Homologene and BLASTP analysis. Any protein that was predicted be nuclear, mitochondrial,
ribosomal, heat shock, transcription/translation-associated or contamination (i.e. keratin, BSA) was not further characterized in this study. The *C. elegans* homologues and paralogs of the 160 remaining proteins were determined from the peptides and subsequently accession/gi numbers retrieved after tandem mass spectrometric analysis. The mammalian accession numbers provided entire protein sequences, which were then used in a BLASTP search of WormPep to determine homology/orthology. Homologues were determined by taking the top scoring sequences (E-value <10^{-10}). They are listed in Table S1. In addition, we double-checked sequences on WormPD (Proteome); (http://www.proteome.com) and Homologene (NCBI); (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=homologene) for homology scores and protein function predictions across species. When two sequences were each other's best match (reciprocal best match), the genes corresponding to the sequences were considered putative orthologs. Paralogs to *C. elegans* genes were previously determined from Wormbase. An additional 11 *C. elegans* genes that had E-value scores between 10^{-9} and 10^{-3} were also included in our screen and are marked by an “*” in the Not Conserved column in Table S1. Two mammalian proteins, Annexin VI and noggin did not have *C. elegans* counterparts. Predicted gene products were systematically placed into functional classes by manual inspection using data from Proteome, Homologene and BLAST analysis. If a mammalian protein had already been shown to play a role in cytokinesis or to localize to the midbody or intercellular canal in *H. sapiens, C. griseus, D. melanogaster, D. discoidium, S. cerevisiae, S. pombe, A. nidulans, A. thaliana, N. tabacum, or X. laevis*, an “X” was marked in the Published Cytokinesis Proteins column (33%, 52/160) and/or in the Previously Localized column (26%, 42/160) in Table S1.

**Immunolocalization and Microscopy**

HeLa cells were grown on 22 X 22 mm cover slips and fixed in 3.7% Formaldehyde, 0.1%
Glutaraldehyde and 0.3% Triton in 1X BRB80 (80mM PIPES pH 6.8, 1mM MgCl2, 1mM EGTA). Immunofluorescence was performed using the following antibodies: DM1A: anti-α-tubulin (ICN Pharmaceuticals), Dynamin II: anti-Dyn2 and Kinesin Heavy Chain: anti-KHC (kind gifts of M. McNiven), RACK1: anti-RACK1 (BD Biosciences), IQGAP1: anti-IQGAP1 (Zymed), KEAP1: anti-Keap1 (kind gift of M. Velichkova and T. Hasson (6), Endoplasm/GRP94: anti-GRP94 (StressGen), RAB-GDI: anti-RabGDI (Zymed), KIF4: anti-KIF4 (kind gift of A. Caceres (7)), Annexin II: anti-Annexin II (BD Biosciences)(Annexin II is the same as Calpactin I Light Chain), BiP: anti-BiP (BD Biosciences), Glut1: anti-Glut1 (H-43): sc-7903 (Santa Cruz Biotechnology, Inc.), V5: anti-V5 epitope (Invitrogen), GM130: anti-GM130 (BD Biosciences). Cells were immunostained with appropriate antibody (see above) and α-tubulin and then mounted in Vectashield with DAPI (Vector Labs). Alexa Fluor® 488 and Alexa Fluor® 568 secondary antibodies (Molecular Probes, Inc.) were used. Visualization was performed on an upright microscope equipped with a laser confocal imaging system (TCS NT; Leica).

**Cell culture and Transfection**

The full-length Novel/CGI-49 DNA sequence (BC026185) was obtained from Open Biosystems and cloned into the pcDNA/V5/GW/TOPO® vector (Invitrogen). The construct was transfected into HeLa cells using the GeneJammer® Transfection Reagent (Stratagene) via manufacture instructions. The cells were stained with anti-tubulin (DM1A) and anti-V5 epitope 3-4 days after transfection.

**Generation of dsRNA**

Primer pairs to specific *C. elegans* genes were obtained from Research Genetics online at (http://www.wormbase.org). If no primer pairs were available, primers to genes were
constructed in AcePrimer (http://elegans.bcgsc.bc.ca/gko/aceprimer.shtml). T7 sequences were added to each primer end to aid in RNA synthesis. It was not unusual that several genes from the Research Genetics primer sequences did not produce products; synthesizing new primers in AcePrimer circumvented this problem. PCR products were concentrated and precipitated using Qiagen MinElute™ PCR Purification Kit (#28006). We synthesized dsRNA using the Ambion T7 Megascript™ High Yield Transcription Kit (#1334). The RNA pellet was diluted in 10 µl of DEPC–treated ddH20, heated to 65°C and cooled on ice. We assessed the quality of the dsRNA by running 1% agarose gels. RNA concentrations varied from ~3-6 mg/ml.

**RNAi screening**

Injection of dsRNA against each gene into a TY3553 hermaphrodite strain (maintained at 25°C) expressing both β-tubulin::GFP and histone H2B::GFP was performed to assess spindle and chromosome defects. Injected hermaphrodites maintained for 24-36 hours at 25°C were cut open to release their progeny and germlines. We recorded 2-3 viable embryos and 4-5 dissected germlines of all viable injected animals using Improvision OpenLab™ software. Data were deposited into the *C. elegans* database, Wormbase (http://www.wormbase.org). Not all embryos observed after RNAi expressed tubulin::GFP brightly. EMB was defined as 10-100% dead embryos. STE/GON was defined as an animal that had a germline cytokinesis defect and was also sterile. STE animals were defined as those that had a brood size of less than 10 (Wild type is 50+) but had no apparent germline cytokinesis defect. See Table S1 legend for complete descriptions.
Supporting Online Tables

The following table is a PDF file:
Table S1

The following table is a PDF file:
Table S2
Table S1:

Mammalian midbody proteins identified, corresponding *C. elegans* genes and their RNAi phenotypes. The mammalian proteins identified by tandem mass spectrometry are listed, followed to the right by the Genbank accession number, the functional group to which it was assigned, the corresponding *C. elegans* gene, and its chromosome location and reported locus. An “X” in columns to the left of the identified protein denotes those factors previously shown to play a role in cytokinesis “Published Cytokinesis Gene” and those previously localized to the midbody or intercellular canal in *H. sapiens*, *C. griseus*, *D. melanogaster*, *D. discoidium*, *S. cerevisiae*, *S. pombe*, *A. nidulans*, *A. thaliana*, *N. tabacum*, or *X. laevis* “Previously Localized”. Phenotypes upon RNAi depletion of each protein in *C. elegans* are noted as: CE: early cytokinesis defects, CL: late cytokinesis defects, STE/GON: sterile (no progeny) with germline cytokinesis defects (i.e. germline membrane organization defects), STE: sterile (no progeny), MITO: mitotic defects (including spindle assembly and cell cycle defects.), MEI: meiosis defects (embryos dead in meiosis and/or polar body extrusion defects), UNC: uncoordinated animals, WT: Wild type (No defects observed), EMB-embryonic lethal. An asterisk in the “Not Conserved” column denotes the mammalian proteins that did not have *C. elegans* homologues, and the 16 *C. elegans* genes showing similarity to the mammalian proteins, with E-value scores between $10^{-9}$ and $10^{-3}$. Note: The underlined phenotypes are those not seen in our analysis but reported elsewhere (See Footnotes in Table S1).
Table S2:

Eliminated mammalian midbody proteins identified, corresponding accession numbers, and their Functional Group. The 417 eliminated mammalian proteins identified by tandem mass spectrometry are listed, followed to the right by the Genbank accession number and the functional group to which it was assigned.
Online Movies

**Movies S1 and S2 correspond to Fig. 4 (see Fig. 4 legend)**

**Movie S1:** Wild type Nomarski

**Movie S2:** K04D7.1-RACK1 RNAi

**Movies S3 and S4 correspond to Fig. 5 (see Fig. 5 legend)**

**Movie S3:** Wild type Histone H2B::GFP

**Movie S4:** T05E11.3-ENDOPLASMIN RNAi in a H2B::GFP background.
Supporting Online References

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Note: The underlined phenotypes represent phenotypes not seen in our analysis but were reported elsewhere.

1 (Kamath et al, 2003)
2 (Simmer et al, 2003)
3 (Kaitna et al, 2002)
4 (Piekny et al, 2002)
5 (Piano et al, 2002)
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<td>Mitochondrial</td>
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<tr>
<td>PROTEINDISULFIDE-ISOMERASE (EC5.3.4.1) ER60 PRECURSOR-HUMAN</td>
<td>CAAB89996.1</td>
<td>Mitochondrial</td>
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<tr>
<td>PR XIV [RATTUS NORVEGICUS]</td>
<td>A26456.1</td>
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<tr>
<td>PYRUVATE DEHYDROGENASE (LIPOAMIDE)(EC1.2.4.1) BETA CHAIN-RAT</td>
<td>P49432</td>
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<tr>
<td>PYRUVATE KINASE (EC2.7.1.40) ISOZYME M1-RAT</td>
<td>P11980</td>
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<td>PYRUVATE KINASE (EC2.7.1.40) ISOZYME M2-HUMAN</td>
<td>AAA36672.1</td>
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<td>Enzyme Name</td>
<td>Accession</td>
<td>Location</td>
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<td>Rat outer mitochondrial membrane cytochrome B5</td>
<td>ICCC_A</td>
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<tr>
<td>Ribose-phosphate pyrophosphokinase (EC2.7.6.1) catalytic chain III-Human</td>
<td>AAB59463.1</td>
<td>Mitochondrial</td>
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<td>Ribose-phosphate pyrophospho kinase I (phospho ribosyl pyro phosphate synthetase) (PRPBP) (PRP-I)</td>
<td>NP_002755.1</td>
<td>Mitochondrial</td>
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<td>Ribosomal protein, mitochondrial L12 [Homo sapiens]</td>
<td>CAAS6249.1</td>
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<td>Serine hydroxy methyl transferase (EC2.1.2) 2-Mouse</td>
<td>CA6A6226.1</td>
<td>Mitochondrial</td>
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<td>Serotransferrin precursor (siderophilin) (beta-1-metal binding globulin)</td>
<td>AAA96731.1</td>
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<td>Similar to dihydrolipoamide S-succinyl transferase (E2 component of 2-oxo-glutarate complex) [Rattus norvegicus]</td>
<td>XP_216753</td>
<td>Mitochondrial</td>
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<td>Succinate dehydrogenase complex, subunit D precursor [Homo sapiens]</td>
<td>AAH09574</td>
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<td>Succinate—co a ligase (GDP-forming) (EC6.2.1.4) alpha chain precursor-Rat</td>
<td>P13086</td>
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<td>Succinate—dehydrogenase FF subunit [Mus musculus]</td>
<td>XP_127445</td>
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<td>Superoxide dimutase 2, mitochondrial [Rattus norvegicus]</td>
<td>P07895</td>
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<td>Thioredoxin [Mus musculus]</td>
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<td>Thioredoxin reductase 1 [Mus musculus]</td>
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<td>Thymidylate synthase (EC2.1.1.45) -Mouse</td>
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<td>Transaminase, glutamate oxaloacetate [Rattus norvegicus]</td>
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<td>Transketolase [Mus musculus]</td>
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<td>Translocase of outer mitochondrial membrane 70 (yeast) homolog A [Homo sapiens]</td>
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<td>Ubiquinol—cytochrome-C reductase (EC1.10.2.2) core protein I-Human</td>
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<td>Ubiquinol—cytochrome-C reductase complex core protein 1 precursor</td>
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<td>Ubiquitin carboxyl-terminal hydrolase 11 (ubiquitin thiolesterase 11) (ubiquitin-specific processing protease 11)</td>
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<td>UDP glucose 6-dehydrogenase (EC1.1.1.22)-bovine</td>
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<td>CB17.2 (RIBOSOMAL PROTEIN S17-LIKE 4) [HOMO SAPIENS]</td>
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<td>RIBOSOMAL PROTEIN L7A (AA 1-266) [RATTUS RATTUS]</td>
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<td>MATERNAL EMBRYONIC MESSAGE 3 [MUS MUSCULUS]</td>
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<td>MATRIN 3 [RATTUS NORVEGICUS]</td>
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<td>NOPP140</td>
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<td>NUCLEAR COREPRESSOR KAP-1 [HOMO SAPIENS]</td>
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<td>NUCLEAR FACTOR OF KAPPA LIGHT POLYPEPTIDE GENE ENHANCER IN B-CELLS 2, P49/P100 [MUS MUSCULUS]</td>
<td>AAD39462.1</td>
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<td>NUCLEAR INHIBITOR OF PROTEIN PHOSPHATASE-1 (NIPP-1) (PROTEIN PHOSPHATASE 1, REGULATORY INHIBITOR SUBUNIT 8) [HOMO SAPIENS]</td>
<td>CA90625.1</td>
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<td>CHAPERONIN CONTAINING TCP1, SUBUNIT 4 (DELTA) [HOMO SAPIENS]</td>
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<td>CHAPERONIN GROEL PRECURSOR - MOUSE</td>
<td>CAA38762.1</td>
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<td>CHAPERONIN SUBUNIT 2 (BETA) [MUS MUSCULUS]</td>
<td>CAA83428.1</td>
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<td>CHAPERONIN SUBUNIT 4 (DELTA) [MUS MUSCULUS]</td>
<td>BAA81875.1</td>
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<td>CHAPERONIN SUBUNIT 5 (EPSILON) [MUS MUSCULUS]</td>
<td>BAA81876.1</td>
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<td>CHAPERONIN SUBUNIT 6A (ZETA) [MUS MUSCULUS]</td>
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<td>CHAPERONIN SUBUNIT 6B (ZETA) [MUS MUSCULUS]</td>
<td>CAA90374.1</td>
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<td>CHAPERONIN SUBUNIT 8 (THETA) [MUS MUSCULUS]</td>
<td>CAA85521.1</td>
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<td>DNAAH HOMOLOG 2 [RATTUS NORVEGICUS]</td>
<td>AAB64094.1</td>
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<td>DNAAH-TYPE MOLECULAR CHAPERONE HSPA5 PRECURSOR - HUMAN</td>
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<td>DNAAH-TYPE MOLECULAR CHAPERONE HSPA6 - HUMAN</td>
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<tr>
<td>FK506 BINDING PROTEIN 4 (59 kDa) [MUS MUSCULUS]</td>
<td>CAA50231.1</td>
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<td>HEAT SHOCK COGNATE 71 kDa PROTEIN</td>
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<td>HEAT SHOCK PROTEIN 20-LIKE PROTEIN [MUS MUSCULUS]</td>
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<td>HEAT SHOCK PROTEIN 47 PRECURSOR - RAT</td>
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<td>HEAT SHOCK PROTEIN HSP 90-ALPHA (HSP 86)</td>
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<td>HEAT SHOCK PROTEIN HSP 90-BETA (HSP 84) (HSP 90)</td>
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<td>HEAT SHOCK PROTEIN HSP27</td>
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<td>HEAT SHOCK PROTEIN, 105 kDa, HSP105 42 C-HSP [MUS MUSCULUS]</td>
<td>AAA99485.1</td>
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<td>HEAT SHOCK PROTEIN, 84 kDa 1 [MUS MUSCULUS]</td>
<td>AAA37865.1</td>
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<td>HEAT SHOCK PROTEIN, 86 kDa 1 [MUS MUSCULUS]</td>
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<td>HEAT-SHOCK COGNATE 70kD PROTEIN (44KD ATPASE N-TERMINAL FRAGMENT) (E.C.3.6.1.3)</td>
<td>1NGJ</td>
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<td>HEPATOCELLULAR CARCINOMA-ASSOCIATED ANTIGEN 57 [HOMO SAPIENS]</td>
<td>AF244136.1</td>
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<tr>
<td>HIRA INTERACTING PROTEIN 4 (DNAJ-LIKE) [HOMO SAPIENS]</td>
<td>CAA04669.1</td>
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<tr>
<td>HSC70-INTERACTING PROTEIN (PROGESTERONE RECEPTOR-ASSOCIATED P48 PROTEIN) (PUTATIVE TUMOR SUPPRESSOR ST13)</td>
<td>CAA10844.1</td>
<td>Heat Shock</td>
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<td>HSP60 PROTEIN (AA 1-547) [RATTUS NORVEGICUS]</td>
<td>A32800</td>
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<td>HYPOTHETICAL PROTEIN FLJ10737 [HOMO SAPIENS]</td>
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<td>ISCHEMIA RESPONSIVE 94 kDa PROTEIN [RATTUS NORVEGICUS]</td>
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<td>P59 PROTEIN (HSP BINDING IMMUNOPHILIN) (HBI) (POSSIBLE PEPTIDYL-PROLYL CIS-TRANS ISOMERASE) (PPIASE) (ROTAMASE)</td>
<td>NP_002005.1</td>
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<td>PRE-MTHSP70 [RATTUS SP.]</td>
<td>AAB33049.1</td>
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<td>STIP1 HOMOLOGY AND U-BOX CONTAINING PROTEIN 1; CARBOXY TERMINUS OF HSP70-INTERACTING PROTEIN [MUS MUSCULUS]</td>
<td>AAD33401.1</td>
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<td>T-COMPLEX PROTEIN 1, ALPHA SUBUNIT (TCP-1-ALPHA) (CCT-ALPHA)</td>
<td>P17987</td>
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<td>TCP1 RING COMPLEX PROTEIN TRIC5 - HUMAN</td>
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<td>ALPHA-1-ANTITRYPsin PRECURSOR - BOVINE</td>
<td>CAA44840.1</td>
<td>Contamination</td>
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<tr>
<td>ALPHA-2-HS-GLYCOPROTEIN PRECURSOR (FETUIN)</td>
<td>CAA34591.1</td>
<td>Contamination</td>
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<td>CHAIN A, BOVINE TRYPsin COMPLEXED WITH RPR128515</td>
<td>BAA07516.1</td>
<td>Contamination</td>
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<td>Chain</td>
<td>Description</td>
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<td>D</td>
<td>Platelet Factor 4</td>
<td>1PLF</td>
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<td>E</td>
<td>Bovine Trypsin (E.C.3.4.21.4) complex with a modified SSI (Streptomyces subtilisin inhibitor) with Met 70 replaced by Gly and Met 73 replaced by Lys (SSI (M70G, M73K))&quot;</td>
<td>3BTW_E</td>
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<td>P</td>
<td>Crystal structure of the thrombin-thrombomodulin complex</td>
<td>IDX5</td>
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<td>S</td>
<td>The crystal structure of modified bovine fibrinogen (AT ~4 angstrom resolution)</td>
<td>NP_476787</td>
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<td>Hemoglobin alpha chain</td>
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<td>Hemoglobin alpha chain [Bos taurus]</td>
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<td>Hemoglobin beta chain - bovine</td>
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<td>Keratin, type II cytoskeletal 1 (cytokeratin 1) (K1) (CK 1) (67 kDa cytokeratin) (hair alpha protein)</td>
<td>NP_006112.1</td>
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<td>Modified beta trypsin (monoisopropylphosphoryl inhibited) (E.C.3.4.21.4) (neutron data)</td>
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<td>Plasmin (EC 3.4.21.7) precursor - bovine</td>
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<td>Serum albumin precursor - bovine</td>
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<td>Thymopoietins beta and gamma (TP beta and TP gamma)</td>
<td>AAB60330.1</td>
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