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H. sapiens      DVEFEVVGDAPEKVGPKQAEDA-AKSITNGSDDGAQPSTS--TAQEQQDDVLIVDSDEEDS 57
M. musculus    DVEFEVVGDSPEKVGPKQAEDA-AKSIANGSDDGAQPSTS--TAQEQQDDVLIVDSDEEGP 57
X. laevis      ---FEVVGDVPEKGPQKPPEES-VKNITNGSDDGAQPSTS--KAQDQDDVLIVDSDEESP 54
D. rerio       ----VVGDAADKAPAPSAPEE-GKNIANGNKDSAQPSTSSKAAVEDDDVLLVDSDEEPS 54
S. cerevisiae  ---CNTCSLPDVEVPLIKANNSPSKNEEEKNEKGADVATTNSHGKDGIVILD-DDEGE 56
D. melanogaster -----DDGPSTSKRSRPNVEVEEDDDCLVIEEDEDQADV VVVVATDKLSVQSPPKSGS 53
                .           . :   . :   :           :           * : : . ..

H. sapiens      SN-NADVSEEEERSRKRKLDEKENLS-AKRSRIEQ-KEELDD--VIALD 100
M. musculus    SN-STDCSGDDKARKRKLEENEAAS-TKKCRLEQ-MEDPDD--VIALD 100
X. laevis      SSSNADVGMEASLRKRKLPDEEAVSSTKRKRIEPPVEEDDD--IALD 100
D. rerio       SS-TMDTESSNRKRKHHDAAETDDAS-SKRKRLDQQPADDDDEDIALD 100
S. cerevisiae  ITIDAEPINGSKKRPVDTEISEAPS-NKRTKLVN---EPTNSDIVELD 100
D. melanogaster KRKPCEVIEDEDITEILESSDDEPAGPTKCRSR-LDDSNPVAVISID 100
                :           .           :   :   . : :   :           :           : : : *

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**Supplemental Figure 1.** Sequence alignment of the C-terminus of Uba2. The C-terminal 100 amino acids of Uba2 from *H. sapiens*, *M. musculus*, *D. rerio*, *X. laevis*, *D. melanogaster* and *S. cerevisiae* were aligned using ClustalW2. Conserved clusters of basic amino acids potentially participating in nuclear import are underlined and highlighted in bold.

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H. sapiens      ---MVEKEEEAGGG---I SEEEAAQYDRQIRLWGLEAQKRLRASRVLLVGLKGLGAEIAK 53
M. musculus    --MVEKEEEAGGGGGGISEEEAAQYDRQIRLWGLEAQKRLRASRVLLVGMKGLGAEIAK 57
D. rerio       MIDTIEKEDT-----I SEEEAAQYDRQIRLWGLEAQKRLRGSRVLLVGLRGLGAEVAK 54
X. laevis      ---MVEKEEA-----V SEEEAAQYDRQIRLWGLEAQKRLRTSRVLLVGMRLGAEVAK 51
D. melanogaster ---MVDMDTSET-AVELTEAENELYDRQIRLWGLEAQKRLRTAKILIAGLCGLGAEITK 56
S. cerevisiae  ---MDMKVEK-----LSEDEIALYDRQIRLWGMTAQANMRSKAVLLINLGAIGSEITK 50
      . :           : * * * * * : * . : * : : * : . : . : * : * : *

H. sapiens      NLILAGVKGLTMLDHEQVTPEDPGAQFLIRTGSVGRNRAEASLERAQNLNPMVDVKVDTE 113
M. musculus    NLILAGVKGLTMLDHEQVSPEDPGAQFLIRTGSVGRNRAEASLERAQNLNPMVDVKVDTE 117
D. rerio       NLILAGVKGLTLLDHEQVTEESRRAQFLIPVDADGQNHAAQASLERAQFLNPMVEVKADTE 114
X. laevis      NLILAGVKALTLDDHEQVSSSEDSRAQFLIPSGSLGQNRAEASLNRRARNLNPVSVVEADTE 111
D. melanogaster NIILSGVNSVKLLDDKDVTEEDFCSQFLVPRESLNTNRAEASLTRARALNPMVDISADRE 116
S. cerevisiae  SIVLSGIGHLTILDGHMVTEDLGSQFFIGSEVDGQWKIDATKERIQDLNPRVELNFDKQ 110
      . : * : * : . : * . : * . : * : * : . : * : * . : . : * : *

H. sapiens      DIEKKPESFFTQFDVAVCLTCCSRDVIKVDQICHKNSIKFFTGDVFGYHGTYFANLG-EH 172
M. musculus    DVEKKPESFFTQFDVAVCLTCCSRDVIKVDQICHRNSIKFFTGDVFGYHGTYFANLG-EH 176
D. rerio       PVESKPDDFFFQFDVAVCLTRCSRDLMVRVDQLCASRNKVFVCGDVYGYNGYMFSDLGQEQY 174
X. laevis      NINQKSDDDFFTQFDVAVCLTSCPSDLLVRVNHICHKHNIKFFTGDVYGYHGSMFADLG-EH 170
D. melanogaster PLKEKTSEFFGQFDVVVNGATNEELLRIDTICRDLGVKFIATDVWGTGFGFYFASLQ-KH 175
S. cerevisiae  DLQEKDEEFFQQFDLVVATEMQIDEAIKINTLTRKLNIPLYVAGSNGLFAYVFIDLI--- 167
      : : * . : * : * * . : : : : . : . . * . * . *

H. sapiens      EFVEEKTQVAVKVSQGVEDGPDTKRAKLDSSSE-----TTMVKKKVVVFCVKEALEV 222
M. musculus    EFVEEKTQVAVKVSQGVEDGPEAKRAKLDSSSE-----TTMVKKKVVLFVFCVKEALEV 226
D. rerio       HYVEEKPKVVKGSNEANDGPEAKKPKIDPNE-----TTMVKKKTI SFCSLKEALEV 224
X. laevis      EFVEEKAKVTKAKPLVEDGPEAKKAKIDPTE-----TILVKKKVVQFCPLKDALEI 220
D. melanogaster SYVEDVINHKVVAN-----SEKKKYYETV-----SIPTQRDVDYPGYSAWLDF 218
S. cerevisiae  EFISEDEKLQSVRPTTVGPISNSRSIIEVTRKDEDEKKTIERIKTKNCYRPLNEVLST 227
      : : : : . : : . : : : . * .

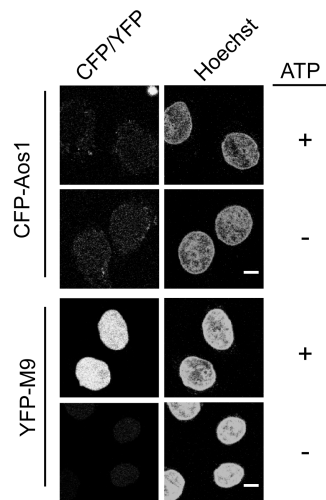
H. sapiens      DWSSEKAKAALKRRTSDYFLLQVLLKFRTDKGRDPSSDYEEDSELLQIRNDVLDLSGI 282
M. musculus    DWSGEKAKAALKRTPADYFLLQVLLKFRTDKGRDPTSESYKEDAELLQIRNDVFDLSGI 286
D. rerio       DWTTEKAKSSLKRIPADYFLLQVLLKFRTDKGRDPQPSFAEDSQQLLQIRDDVLETMGL 284
X. laevis      DWRSEKAKSALKKTPDYFLLQVLMKFRTDKGRDPQPSYQEDSELLQICSDVLDLSLGV 280
D. melanogaster DVTEPSYLRKLRNGPGVLLSVLQKFRTHKRDPSYKTREADLELLRGIREDLPLNS-- 276
S. cerevisiae  ATLKEKMTQRQLKR-VTSSILPLTSLILQYDLNQGKKAISFEQMKRDAAVWCENLGPATV 286
      . :           : * . * : : . : . : . :

H. sapiens      SPDLLPEDFVRYCFSEMAPVCAVVGGLAQQEIVKALSQRDPHNNFFFFDGMKNGIVEC 342
M. musculus    SPDLLPDDFVRYCFSEMAPVCAVVGGLAQQEIVKALSQRDPHNNFFFFDGMKNGIVEC 346
D. rerio       SSDLLPNTFVSYCFSEMSPVCAVVGGLGQEIIVKALSQRDAPHRNFFFFDGLKGSVVYD 344
X. laevis      SPDLLPKDFASYCFSEMAPVCAVVGGLGQEIIVKALSRLDAPHRNFFFFDGTSGIVDC 340
D. melanogaster ---ILGDEALGLIFAQISPAVAVVGVAQEVIVKVVTKLEAPHRNLFVFDPETCAGYVEA 333
S. cerevisiae  VKDDYVQQFIKQKGI EFAPVAAIIGGAVAQDVINILGKRSLPLNFI VFDGITLDMPLFE 346
      . :           : : * . * : * : : : . * . : * : * . :

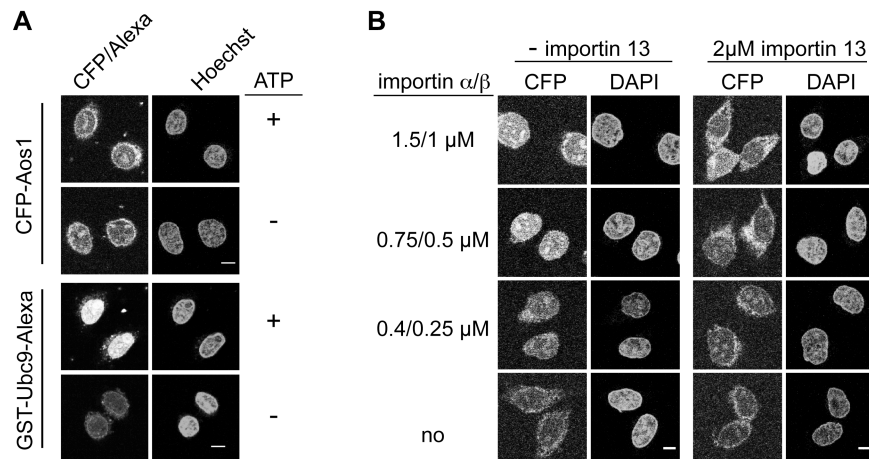
H. sapiens      LGPK 346
M. musculus    LGPQ 350
D. rerio       FSSK 348
X. laevis      LGSK 344
D. melanogaster IGAK 337
S. cerevisiae  F--- 347
      :

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**Supplemental Figure 2.** Sequence alignment of Aosl. Amino acid sequences of Aosl from *H. sapiens*, *M. musculus*, *D. rerio*, *X. laevis*, *D. melanogaster* and *S. cerevisiae* were aligned using ClustalW2. Conserved clusters of basic amino acids potentially participating in nuclear import are underlined and highlighted in bold.

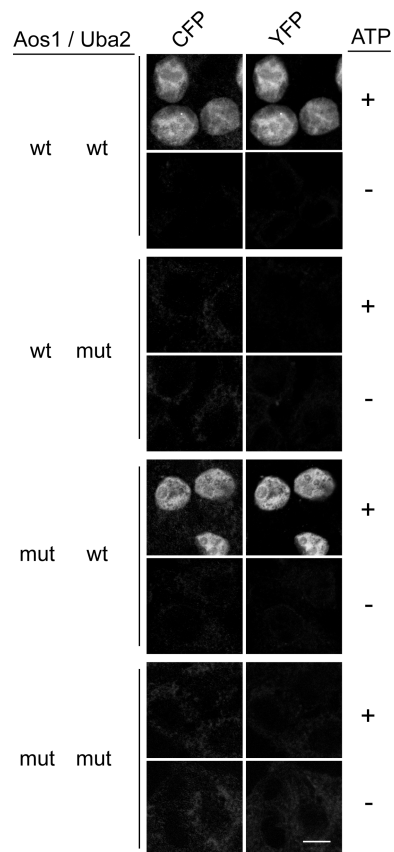


**Supplemental Figure 3.** Transportin does not mediate import of CFP-Aos1 *in vitro*. *In vitro* import of CFP-Aos1 (1 $\mu$ M) in semipermeable HeLa cells was tested in the presence of Transportin (1 $\mu$ M) and 12  $\mu$ M Ran with or without ATP. The transportin dependent cargo YFP-M9 (1 $\mu$ M) (Siomi and Dreyfuss, 1995; Nakielny et al., 1996) was included as a positive control. Nuclear accumulation of fluorescently labelled cargo proteins was analyzed by confocal microscopy. Bar, 10  $\mu$ m.

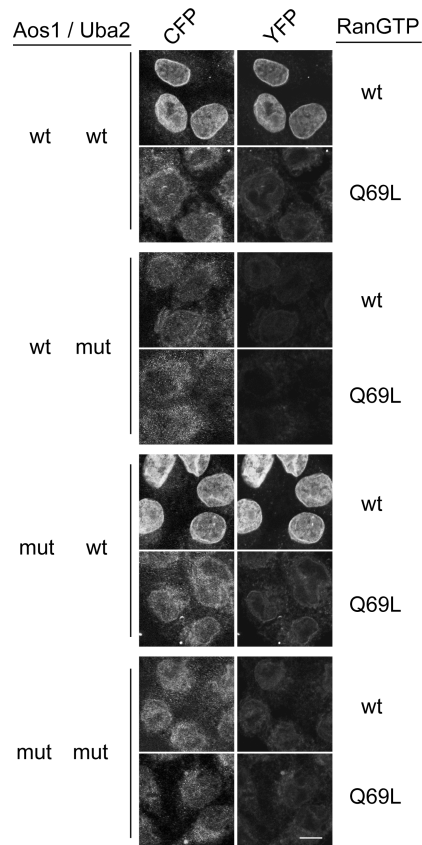


**Supplemental Figure 4.** Importin 13 does not support import of CFP-Aos1 *in vitro*.

(A) CFP-Aos1 (5  $\mu$ M) import was tested in semi-permeabilized HeLa cells in the presence of importin 13 (1  $\mu$ M) and Ran (12  $\mu$ M) with or without ATP. Alexa488 labelled Gst-Ubc9 (Mingot et al., 2001) served as a positive control. Nuclear accumulation of CFP-Aos1 and Gst-Ubc9-Alexa488 was analyzed by fluorescence microscopy. Bar, 10  $\mu$ m. (B) Importin 13 does not synergize with importin  $\alpha/\beta$  for the import of CFP-Aos1. *In vitro* import of CFP-Aos1 (1 $\mu$ M) using semipermeable HeLa cells was performed with the indicated concentrations of Importin  $\alpha$  and importin  $\beta$  in the presence of Ran (12 $\mu$ M), with or without addition of 2  $\mu$ M importin13. Nuclear accumulation of CFP-Aos1 was analyzed by confocal microscopy. Bar, 10 $\mu$ m.



**Supplemental Figure 5.** The Uba2 NLS is required and sufficient for import of the SUMO E1 holo-enzyme. *In vitro* import of E1 complexes reconstituted from wild type proteins (CFP-Aos1-wt, Uba2-wt-YFP) and/or NLS mutants (CFP-Aos1-KR195,196A<sub>2</sub>, Uba2-KR623,624A<sub>2</sub>-YFP). The assays were performed in semipermeabilized HeLa cells in the presence of cytosol and ATP-regenerating system (+). Control experiments contained ATP-depleting system (-) instead. Nuclear accumulation of CFP-Aos1 and Uba2-YFP was analyzed by fluorescence microscopy. Bar, 10  $\mu$ m.



**Supplemental Figure 6.** Importin  $\alpha/\beta$  mediates *in vitro* import of the SUMO E1 holo-enzyme via interaction with the Uba2 NLS. *In vitro* import of E1 complexes reconstituted from wild type proteins (CFP-Aos1-wt, Uba2-wt-YFP) and/or NLS mutants (CFP-Aos1-KR195,196A<sub>2</sub>, Uba2-KR623,624A<sub>2</sub>-YFP). The assays were performed in semi-permeabilized HeLa cells in the presence of importin  $\alpha/\beta$  in the presence of wt Ran or RanQ69L. Nuclear accumulation of CFP-Aos1 and Uba2-YFP was analyzed by fluorescence microscopy. Bar, 10  $\mu$ m.