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ORIGINAL PAPERS

Drosophila caliban, a nuclear export mediator, can function as a tumor suppressor in human lung cancer cells

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We previously showed that the *Drosophila* DNA binding homeodomain of Prospero included a 28 amino-acid sequence (HDA) that functions as a nuclear export signal. We describe here the identification of a protein we named Caliban, which can directly interact with the HDA. Caliban is homologous to human Sdccag1, which has been implicated in colon and lung cancer. Here we show that Caliban and Sdccag1 are mediators of nuclear export in fly and human cells, as interference RNA abrogates export of EYFP-HDA in normal fly and human lung cells. Caliban functions as a bipartite mediator nuclear export as the carboxy terminus binds HDA and the amino terminus itself functions as an NES, which directly binds the NES receptor Exportin. Finally, while non-cancerous lung cells have functional Sdccag1, five human lung carcinoma cell lines do not, even though Exportin still functions in these cells. Expression of fly Caliban in these human lung cancer cells restores EYFP-HDA nuclear export, reduces a cell's ability to form colonies on soft agar and reduces cell invasiveness. We suggest that Sdccag1 inactivation contributes to the transformed state of human lung cancer cells and that Caliban should be considered a candidate for use in lung cancer gene therapy.
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Introduction

Prospero (Prox in mammals) is a homeodomain transcription factor that is required for patterning the *Drosophila* nervous system (Doe *et al.*, 1991; Vaessin *et al.*, 1991; Matsuzaki *et al.*, 1992). Prospero's 64 amino-acid homeodomain (HD) and adjacent 100 amino-acid Prospero domain are highly conserved from flies to man (Burglin, 1994; Demidenko *et al.*, 2001; Ryter *et al.*, 2002). Together they constitute one functional domain controlling DNA binding and nuclear export, and resembling a heterodimeric homeodomain (Demidenko *et al.*, 2001; Ryter *et al.*, 2002; Bi *et al.*, 2003; Yousef and Matthews, 2005).

We previously demonstrated that a 28 amino-acid region from the beginning of Prospero's homeodomain (HDA) functions as an Exportin-dependent nuclear export signal (NES). The intact homeo/Prospero domain is postulated to regulate nuclear export by inhibiting or masking the NES in most cell types. This was suggested by the identification of two *prospero* mutations that result in Prospero protein accumulation in the cytoplasm of embryonic *Drosophila* cells that normally have nuclear protein (Demidenko *et al.*, 2001; Bi *et al.*, 2003).

Exportin functions as a receptor for cargo proteins that contain a short leucine-rich sequence (Wen *et al.*, 1995; Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Stade *et al.*, 1997). Exportin signals typically consist of specifically spaced leucine or hydrophobic amino acids, $LX_{1-3}LX_{2-3}LXL$ (Henderson and Eleftheriou, 2000). The HDA export sequence also consists of conserved hydrophobic residues; however, the spacing, LX_4LX_4LXF (Bi *et al.*, 2003), does not match that of a canonical Exportin signal. The tertiary structure of the homeo/Prospero domain places the nuclear export signal on the outside of the protein, covered or masked only by the Prospero domain (Ryter *et al.*, 2002; Bi *et al.*, 2003; Yousef and Matthews, 2005). If this surface was exposed by an as yet unidentified unmasking function, it would reveal an unusually large hydrophobic surface (Bi *et al.*, 2003). This suggests that when unmasked, the export signal might interact with other proteins to regulate its function. Here we use the homeodomain as bait in a yeast two-hybrid screen (Fields and Song, 1989) to identify a protein that regulates HDA nuclear export, which we have named Caliban (Clbn).

Clbn is homologous to the human NY-CO-1 or Sdccag1 (serologically defined colon cancer antigen gene) protein, which was first identified by a serological analysis of recombinant cDNA expression libraries

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(SEREX), where it was recognized by autologous serum from colon-cancer patients. The carboxy terminal 366 amino acids of this protein were believed to constitute the full-length protein (Scanlan *et al.*, 1998). Another group identified the protein as a tumor suppressor, as when *sdccag1* was induced in a non-small-cell lung cancer cell line, NSCLC-N6, it caused a cell cycle arrest. Blocking the synthesis of Sdccag1 protein with antisense RNA reverted the cells to cancer (Carbonnelle *et al.*, 2001).

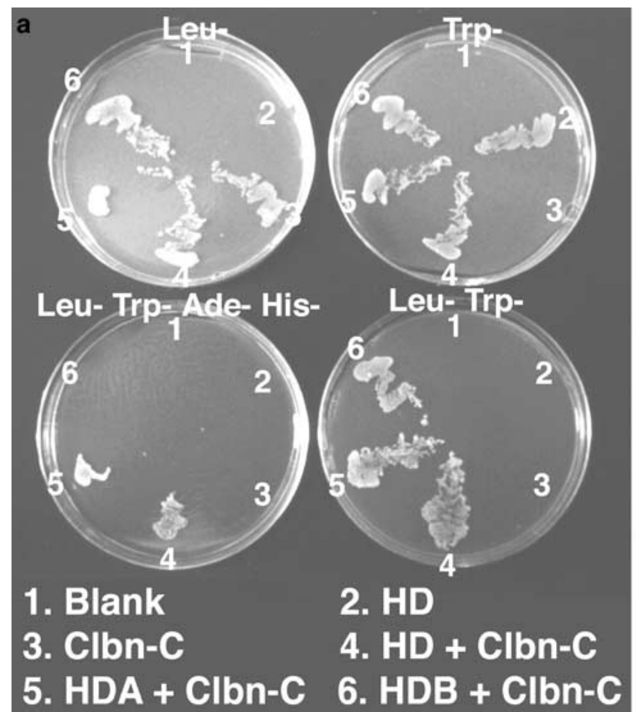
In this paper, we demonstrate that both fly Clbn and human Sdccag1 function as nuclear export mediators of the EYFP-HDA fusion protein. Using EYFP-HDA as a biomarker, we demonstrate that, while normal human lung cells express functional Sdccag1, five human lung cancer cell lines lack Sdccag1 function. Finally, expressing fly Clbn in these human cancer cells demonstrates marked tumor suppression.

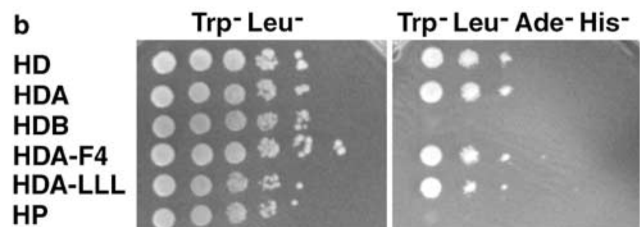
Results

Clbn regulates nuclear export directed by HDA

To identify proteins that might regulate HDA nuclear export function, we used the homeodomain from Prospero as bait in a yeast two-hybrid screen. One gene was independently cloned 35 times. All 35 clones included the carboxy terminal 200 amino acids of Clbn (Clbn-C). To test whether Clbn specifically interacts with the HDA nuclear export signal, yeast were left untransformed (Blank) or transformed with the indicated clones (Figure 1a). Co-transformation of Clbn-C and HD or HDA allows yeast to survive on selective media, indicating a protein–protein interaction. The remainder of the homeodomain, HDB, does not interact with Clbn-C (Figure 1a).

To examine the relative strength of the protein interactions we plated a dilution series of yeast co-transformed with the Clbn-C construct used in Figure 1a and the indicated HD constructs (Figure 1b). The interactions between Clbn-C and HD or HDA are indistinguishable. Two mutant forms of HDA, HDA-F4 and HDA-LLL, which abrogate much of its nuclear export function (Bi *et al.*, 2003), reduce their interactions with Clbn-C approximately fivefold (Figure 1b). The homeo/Prospero domain (HP), which when intact masks nuclear export, does not interact with Clbn-C in yeast. The failure to detect an interaction between Clbn-C and HDA in the context of the intact HP suggests that nuclear export signal masking might be responsible for blocking this interaction. There is no





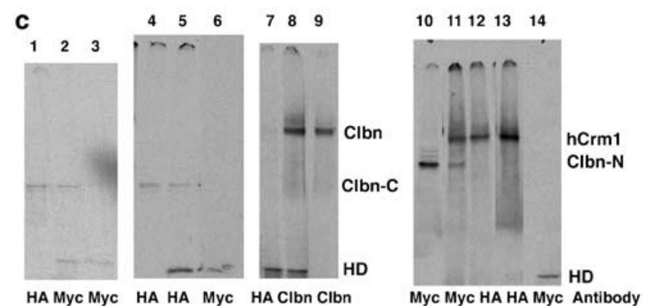


Figure 1 Clbn binds a functional nuclear export signal. (**a**) Yeast (AH109) were transformed with the indicated clones and streaked onto selective media as shown. The carboxy terminus of Clbn-C interacts with HD and HDA but not with HDB. (**b**) The interaction of Clbn and the NES is impaired by mutations that disrupt nuclear export. Yeast were co-transformed with Clbn-C and the indicated clones, and plated on selective media at 10-fold serial dilutions. Relative survival under stringent selective conditions indicates the interaction strengths of the expressed proteins. HDA-F4 and HDA-LLL express mutant versions of the NES; HP expresses a masked NES (Bi *et al.*, 2003). (**c**) The carboxy and amino termini of Clbn bind the HD nuclear export signal and Exportin, respectively. Clones were *in vitro* transcribed and translated in the presence of ^{35}S-methionine, mixed and incubated as indicated, and immunoprecipitated with the indicated antibodies. HA-Cbln-C (lane 1), HA-Clbn-C and Myc-HD (lane 2), Myc-HD (lane 3), HA-Clbn-C (lane 4), HA-Clbn-C and Myc-HD (lane 5), Myc-HD (lane 6), HA-HD (lane 7) HA-HD and Myc-Clbn (lane 8), Myc-Clbn (lane 9), Myc-Clbn-N (lane 10), Myc-Clbn-N and HA-Exportin (lane 11), HA-Exportin (lane 12), Myc-HD and HA-Exportin (lane 13), Myc-HD (lane 14). RanQ69LGTP (Bischoff *et al.*, 1994) was added to the samples used in lanes 11 and 13

interaction between Clbn-C and HDB. These interactions in yeast parallel our earlier analysis of HDA sequence requirements for *in vivo* NES function (Demidenko *et al*., 2001; Bi *et al*., 2003) and suggest that Clbn functions as a regulator of HDA-directed nuclear export.

Clbn is conserved from yeast to man with the fly (NM_143084.1) and human (NM_004713.2) sequences being 49% identical (Figure 2). While the fly gene was predicted to contain five introns, using RT–PCR we were never able to see the predicted 60-nucleotide second intron spliced out of the mature mRNA (data not shown). Inclusion of the resulting additional 20 amino acids improves the alignment of the fly and human protein sequences. There are three regions of higher homology ($\geqslant$50% identity with fewer gaps). These include the amino-terminal 206 amino acids, which are 56% identical. This region is also rich in conserved leucine residues, with 19 of the 26 leucines found in the fly protein also in identical positions of the human protein and four others having conservative changes (Figures 2 and 3a). A second region of increased similarity includes amino acids 277–679, which are 62% identical. A third region includes the carboxy-terminal 179 amino acids, the region that binds HDA, which are 51% identical between flies and man.

Human Sdccag1 has been implicated in colon and lung cancers, but its biochemical function was unknown (Scanlan *et al*., 1998; Carbonnelle *et al*., 2001). To determine the biological function of the fly homolog Clbn, we transfected *Drosophila* SL2 tissue culture cells with a green fluorescent tagged NES, pAc5.1-EYFP-HDA, and used RNAi to determine whether Clbn and/or the *Drosophila* Exportin protein, encoded by *embargoed* (*emb*; Collier *et al*., 2000), are necessary for HDA-mediated nuclear export. While double-stranded vector RNA had no effect on EYFP-HDA localization and EYFP RNAi eliminated green fluorescent protein expression (Figure 3b and data not shown), both *clbn* and *emb* RNAi abrogated nuclear export (Figure 3c–d). The effect of Exportin RNAi is specific to fly Emb, as human Exportin (hCrm1) RNAi did not alter the localization of EYFP- HDA, even following three successive rounds of RNAi treatment (data not shown). We conclude that both Clbn and Emb are required for HDA to function as a nuclear export signal in *Drosophila*.

It was enigmatic why Sdccag1 was cloned using SEREX (Scanlan *et al*., 1998), which implies that it is over- or mis-expressed in colon cancer patients, if it is in fact a human tumor suppressor (Carbonnelle *et al*., 2001). As the former paper described their clone of the carboxy-terminal third of Sdccag1 as full length, we reasoned that this truncated protein might function as a dominant negative. To test this, we co-transfected SL2 cells with pAc5.1-EYFP-HDA and either the amino or carboxy terminal third of Clbn, Clbn-N or Clbn-C, respectively (Figure 3a). While expression of Clbn-N had no effect on the subcellular localization of EYFP-HDA (data not shown), expression of Clbn-C abrogated nuclear export, demonstrating that it acts as a dominant negative (Figure 3e).

Clbn is a bipartite mediator of nuclear export

The amino-terminus of Clbn is enriched in conserved leucine residues, suggesting that it might include one or more nuclear export signals (Figure 3a). To test this we fused the amino-terminal 435 amino acids of Clbn to green fluorescent protein and expressed the constructs in mammalian cells. While pEYFP-Clbn1/435 is ubiquitous, deletion of the amino-terminal 50 amino acids, pEYFP-Clbn51/435, results in the protein accumulating in the cytoplasm (Figure 3f, g). Thus like Prospero, Clbn may have a nuclear export signal-masking domain. Further deletions to amino acid 91 or 131, pEYFP-Clbn91/435 and pEYFP-Clbn131/435, also resulted in the protein accumulating in the cytoplasm; however, deletion to amino-acid 181 partially restored nuclear green fluorescent protein (Figure 3h–j). Deletion to amino-acid 311, pEYFP-Clbn311/435, results in a fusion protein that is not excluded from the nucleus (Figure 3k). Thus Clbn has a minimum of two nuclear export signals, one included within amino-acids 131–181 and the other 181–311.

As the carboxy terminus of Clbn binds HDA in yeast (Figure 1a, b), the amino-terminus functions as an NES (Figure 3f–k), and Clbn is required for the nuclear export of an HDA fusion protein (Figure 3b–e), we reasoned that Clbn might directly mediate the nuclear export of HDA. Epitope-tagged versions of the carboxy terminus of Clbn and HD were synthesized by *in vitro* transcription and translation in the presence of ^{35}S-methionine. When incubated together, immunoprecipitation of HD or Clbn-C resulted in the precipitation of the other protein (Figure 1c, lanes 2 and 5). This interaction does not require RanGTP, a necessary component for Exportin binding to NES sequences (Fornerod *et al*., 1997). Immunoprecipitation of *in vitro* synthesized full-length Clbn with a Clbn peptide antibody was also able to precipitate HD (Figure 1c, lane 8).

Epitope-tagged versions of the amino-terminus of Clbn (Clbn-N) and Exportin, encoded by the human *crm1* gene (Fornerod *et al*., 1997), were synthesized *in vitro*, incubated together in the presence of the nonhydrolizable RanQ69LGTP (Bischoff *et al*., 1994) and immunoprecipitated with Myc-tagged Clbn-N, resulting in the precipitation of Exportin (Figure 1c, lane11). A similar experiment conducted with HD and Exportin, in the presence of RanQ69LGTP, showed that immunoprecipitation of Exportin fails to or only weakly precipitates HD (Figure 1c, lane 13). Thus, Clbn functions as a bipartite mediator of nuclear export, with the amino-terminus binding the nuclear export machinery and the carboxy terminus binding the nuclear export signal HDA.

Sdccag1 is inactive in lung cancer cells

Mutation of *sdccag1* had been implicated in colon and lung cancer. As a biomarker assay for Sdccag1 function,

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MKSRFSTIDL RAVLAELNAS LLGMRVNNVY DVDNKTYLIR LQK--PDFKA TLLLESGIRI HT 60 Hs
**^**^*^*^    ^***    *^* *** ^* ******** * ^*      *  ***^*** *  **
MKTRFNTFDI ICGVAELQ-K LVGWRVNQIY DVDNKTYLFR MQGTGAVEKV TLLIESGTRF HT 61 Dm

TEFEWPKNMM PSSFAMKCRK HLKSRRLVSA KQLGVDRIVD FQFGSDEAAY HLIIELYDRG 120
* *******  **** ** ** ***^^**    ^*^* ***** ****^ ^*** *^*^******
TRFEWPKNMA PSGFSMKLRK HLKNKRLEKV QQMGSDRIVD FQFGTGDAAY HVILELYDRG 121

NIVLTDYEYV ILNILRFRTD EADDVKFAVR ERYPLDHARA AEPLLTLERL TEIVASAPKG 180
*^^*****    * ***  *  * ^ ^^**^* *^**^^ *^      * ****   ^^ ^*  *
NVILTDYELT TLYILRPHT- EGENLRFAMR EKYPVERAKQ PTKELELEAL VKLLENARNG 180

ELLKRVLNPL LPYGPALIEH CLLENGFSGN V--------- ---------- -------KVD 214
^ *^^^* *  *  ***^***  **  *^    *                             ^
DYLRQILTPN LDCGPAVIEH VLLSHGLDNH VIKKETTEET PEAEDKPEKG GKKQRKKQQN 240

EKLETK---- --DIEKVLVS LQKAEDYMKT TSNFSGKGYI IQKREIKPCL EADKPVEDIL 268
 *** *       *^   ^   ^^ * ^ ^    ^^   **** ** ^* **        **
TKLEQKPFDM VNDLPILQQA VKDAQELIAE GNSGKSKGYI IQVKEEKPT- -ENGTVEFFF 290

TYEEFHPFLF SQHSQCPYIE FESFDKAVDE FYSKIEGQKI DLKALQQEKQ ALKKLDNVRK 328
   *******  *         ****  **** ***  * *** *^* ****^  ***** **^
RNIEFHPYLF IQFKNFEKAT FESFMEAVDE FYSTQESQKI DMKTLQQERE ALKKLSNVKN 358

DHENRLEALQ QAQEIDKLKG ELIEMNLQIV DRAIQVVRSA LANQIDWTEI GLIVKEAQAQ 388
**  *** *  ^ *^^*^ *  ***  *  ^* * **^ *^** ^*^*^ * ^*   ^******
DHAKRLEELT KVQDVDRKKA ELITSNQSLV DNAIRAVQSA IASQLSWPDI HELVKEAQAN 418

GDPVASAIKE LKLQTNHVTM LLRNPYLLSE EEDDDVDGDV NVEKNETEPP KGKKKKQKNK 448
** *** **  *** ***^^^ ^*  **  ^^ *^**  * ^* ^*
GDAVASSIKQ LKLETNHISL MLSDPY-DND EDDDLKDPEV TV-------- ---------- 459

QLQKPQKNKP LLVDVDLSLS AYANAKKYYD HKRYAAKKTQ KTVEAAEKAF KSAEKKTKQT 508
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---------- --VDVDLALS AWANARRYYD MKRSAAQKEK KTVDASQKAL KSAERKTQQT 507

LKEVQTVTSI QKARKVYWFE KFLWFISSEN YLIIGGRDQQ QNEIIVKRYL TPGDIYVHAD 568
****^*^^^*  *****^*** ** ******* **^***** * ***^*****^  * ******^
LKEVRTISNI VKARKVFWFE KFYWFISSEN YLVIGGRDAQ QNELIVKRYM RPKDIYVHAE 567

LHGATSCVIK NPTGEPIPPR TLTEAGTMAL CYSAAWDARV ITSAWWVYHH QVSKTAPTGE 628
^ **^* ^*^ ***** ***^ ** ***^**^  **^****^* ^*^  **    **********
IQGASSVIIQ NPTGEEIPPK TLLEAGSMAI SYSVAWDAKV VTNSYWVTSD QVSKTAPTGE 627

YLTTGSFMIR GKKNFLPPSY LMMGFSFLFK VDESCVWRHQ GERKVRVQDE DMETLASCTS 688
** ******* *******    * ** * *** ^^^* ^ **  ******  ^^ *        ^
YLATGSFMIR GKKNFLPSCH LTMGLSLLFK LEDSFIERHL GERKVRSLED DQIDPNVKEN 687

ELISEEMEQL DGGDTSSDED KEEHETPVEV ELMTQVDQED ITLQSGRDEL NEELIQEESS 748
*^         ^    *  ^*         *     * ^      *    ^ *      *   *
EV-------- EHDLLSDNED ADSNINLSEP SSNTEITAFP NTEV--KIEH DTGRIIVRSD 737

EDEGEYEEVR KDQDSVGEMK DEGEETLNYP DTTIDLSHLQ PQRSIQKLAS KEESSNSSDS 808
    * ** ^      ^  ^     ^^     ^*** *
SVNPEIEETK ESEVVLDKIL KKTDDE---- ETTIILA--- ---------- ---------G 771

KSQSRRHLSA KERREMKKKK LPSDSGDLEA LEGKDKEKES TVHIETHQNT SKNVAAVQPM 868
 *^ ^^     *   ^  ^ ^       ^^   ^    *
PSRKKQVSAK KTKEDKARAK QEAAKQEVPP VSSEPKNPSQ V--------- ---------- 812

KRGQKSKMKK MKEKYKDQDE EDRELIMKLL GSAGSNKEEK GKKGKKGKTK DEPVKKQPQK 920
***** *^** ** ******^ *^**^ * ^*  * *  *                     **
KRGQKGKLKK MKQKYKDQDD EEREIRMMIL KSSGKEK--- ---------- -------PQA 852

PRGGQRVSDN IKKETPFLEV ITHELQDFAV DDPHDDKEEQ DLDQQGNEEN LFDSLTGQPH 988
             **    *           ^ **  ^      *^*        ^  *******
SADKVVEKSE STKEYVKPEK SAAPKNPVEL DDADEVPVGG DVD------- VLNSLTGQPH 905

PEDVLLFAIP ICAPYTTMTN YKYKVKLTPG VQKKGKAAKT ALNSFMHSKE ATAREKDLFR 1048
  * ****** ^ ***  ^ * **^*******   *^*****  *** *   *   ^****** ^
EGDELLFAIP VVAPYQALQN YKFKVKLTPG TGKRGKAAKL ALNIFAKEKS CSAREKDLLK 965

SVKDTDLSRN IPGKVKVSAP NLLNVKRK 1076
*^*^  *  ** ******^***  *    ^
SIKEESLARN IPGKVKLSAP QLQKYHK   992
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Figure 2 Alignment of human (Hs) and fly (Dm) Clbn/Sdccag1 protein sequences. The human (NM_004713.2) and fly (NM_143084.1) sequences were aligned with identical residues indicated by * and conserved changes by ˆ. The fly sequence includes the additional 20 amino acids previously thought to be a second intron

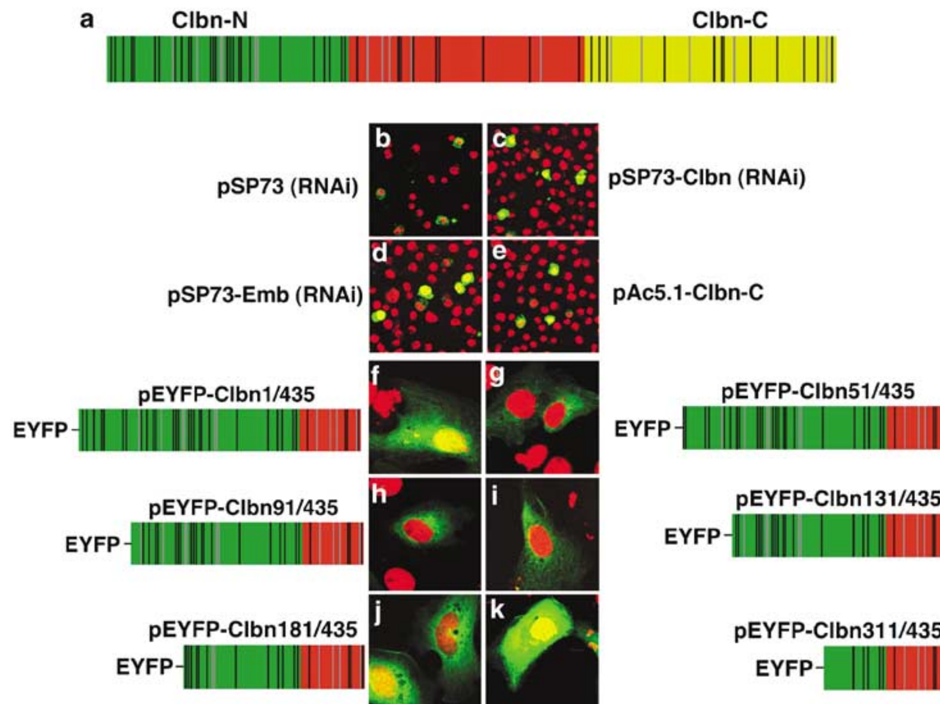

Figure 3 Clbn is required for the nuclear export of HDA and itself contains functional NESs. (**a**) Schematic diagram of Clbn with leucine residues conserved between man and fly shown as black bars and conservative substitutions shown as gray bars. (**b–k**) Cells were fixed, stained with propidium iodide and examined by confocal microscopy as described elsewhere (Demidenko *et al*., 2001); EYFP fusion protein is shown in green, DNA in red and overlap in yellow. (**b–e**) *Drosophila* SL2 tissue culture cells were cotransfected with a clone expressing the green-fluorescent-tagged nuclear export signal, pAc5.1-EYFP-HDA, and RNAi synthesized from the vector pSP73 (**b**), pSP73-Clbn (**c**), or pSP73-Emb (**d**), or with the plasmid pCMV-Clbn-C (**e**). (**f–k**), Mammalian CV1 cells were transfected with plasmids expressing a deletion series of the amino-terminal 435 amino acids of Clbn fused to green fluorescent protein. (**f**) Cbln1/435, (**g**).Clbn51/435, (**h**) Clbn91/435, (**i**) Clbn131/435, (**j**) Clbn181/435, (**k**) Clbn311/435

we transfected pEYFP-HDA into various human cancer cell lines and examined their ability to export green fluorescent fusion protein from the nucleus. While the colon cancer cell lines HCT-116 and SW620 were able to export EYFP-HDA (data not shown), all five lung-cancer cell lines that were tested failed to show export (Figure 4a and data not shown). We confirmed that Exportin still functions in these cells by using a green fluorescent fusion protein, pREV (1.4)-GFP-PKI (Henderson and Eleftheriou, 2000). The nuclear export signal of protein kinase A inhibitor (PKI) directly binds Exportin and is a functionally strong nuclear export signal (Wen *et al*., 1995; Fornerod *et al*., 1997; Henderson and Eleftheriou, 2000). We observed nuclear export of REV (1.4)-GFP-PKI in all the cell lines (Figure 4a). Furthermore, nuclear import also functions in these cells as demonstrated by the nuclear localization of EYFP-Pros-NLS+, a fusion protein that includes the Prospero NLS and a masked version of the NES (Figure 4a).

It was possible that *sdccag1* is not normally expressed in lung tissue. To test this, we probed a Northern blot consisting of poly A^+ RNA from different human tissues. A 4.4 kb full length transcript was detected in brain, heart, liver, lung, spleen and skeletal muscle tissue; significantly less signal was seen in stomach and testis (Figure 4b).

To determine whether Sdccag1 functions in lung cells, we transfected a primary normal human bronchial epithelial cell line (NHBE) and the immortal 'normal' lung cell lines WI-38 and IMR-90 with pEYFP-HDA, pREV(1.4)-GFP-PKI and pEYFP-Pros-NLS+. All three cell lines showed nuclear export of the green fluorescent proteins fused to HDA or PKI and nuclear localization of EYFP-Pros-NLS+ (Figure 4a, data not shown). Thus, Sdccag1 functions in normal lung cell lines; however, it appears to be inactive in all five lung cancer cell lines investigated.

To determine if inactivation of *sdccag1* is solely responsible for the change in HDA subcellular localization, we inactivated *sdccag1* in the normal lung cell lines NHBE and WI-38 and expressed fly Clbn in the lung cancer cell lines A549 and EKVX. First, in normal lung cells, we stably integrated two different clones, pSM2c-20 and pSM2c-21, that express short hairpin RNAs (shRNA) from regions that encode the amino-terminus of Sdccag1. These cells were then transfected with pEYFP-HDA and shown to no longer export the HDA fusion protein from the nucleus (Figure 4c and data not shown). These cells continue to export REV (1.4)-GFP-PKI and localize EYFP-Pros-NLS+ to the nucleus (data not shown). Thus, as with fly cells, Sdccag1 function is required in mammalian cells for the export of HDA fusion proteins.

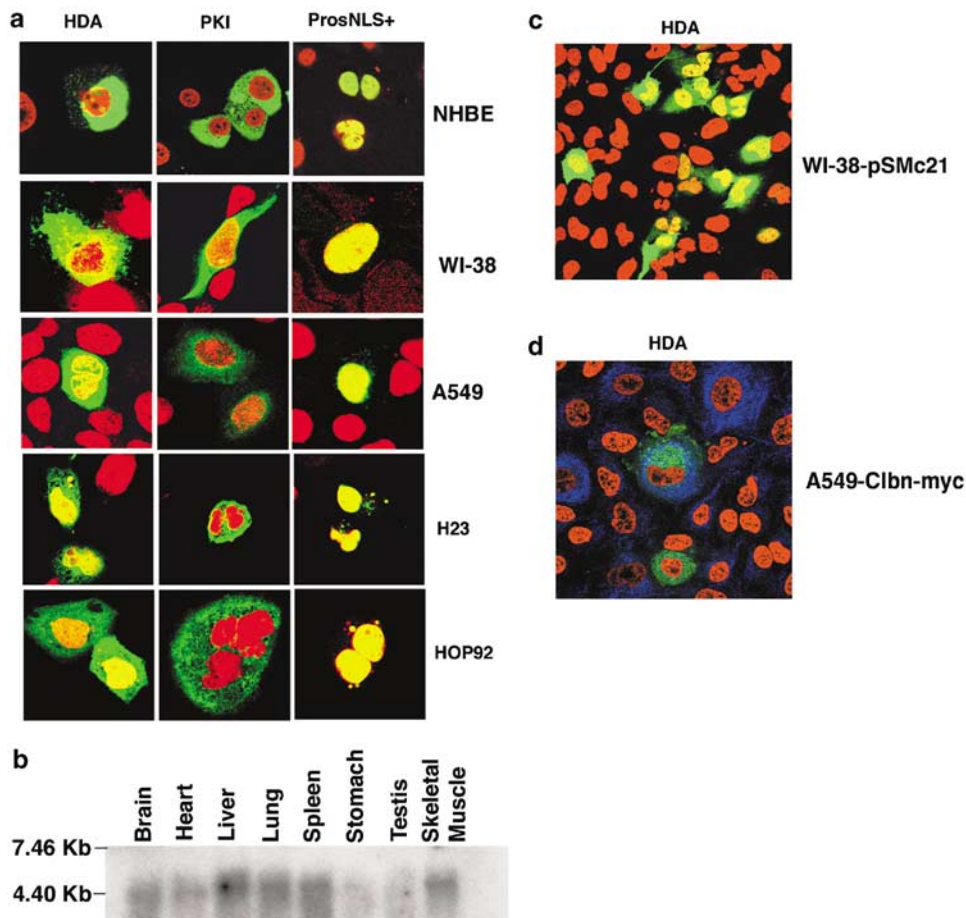

Figure 4 Sdccag1 is inactive in lung cancer cell lines. (**a**) Human lung cell lines were transfected with pEYFP-HDA (HDA), pREV(1.4)-GFP-PKI (PKI) or pEYFP-Pros-NLS+ and fixed, stained and examined as in Figure 3. A primary NHBE and two immortal 'normal' embryonic lung cell lines, WI-38 and IMR-90 (not shown), were compared to five lung cancer cell lines including A549, H23 and HOP92 (HOP62 and EKVX not shown). (**b**) *sdccag1* RNA is expressed in most tissues. (**c**) *sdccag1* RNAi abrogates nuclear export of EYFP-HDA in WI-38 cells. (**d**) Expression of fly Clbn in A549 cells is sufficient to allow nuclear export of EYFP-HDA. Clbn is shown in blue. Clbn expression and export of EYFP-HDA are independent of the addition of teteracycline, indicating that the repressor is not working efficiently in any of the extant cell lines

Second, in the lung cancer cell lines A549 and EKVX, we stably integrated a plasmid that expresses the full-length fly *clbn* gene. These cells were then transfected with pEYFP-HDA and shown to have restored ability to export EYFP-HDA, as both the fusion protein and a myc-tagged Clbn protein accumulate in the cytoplasm of lung cancer cells (Figure 4d and data not shown). We conclude that HDA fusion proteins can be used as effective biomarkers to detect Clbn/Sdccag1 function and that Sdccag1 is nonfunctional in all five nonsmall cell lung cancer cell lines that we examined.

Fly Clbn can act as a tumor suppressor in human lung cancer cells

Does *sdccag1* inactivation play a role in lung cancer tumorigenesis? In order to answer this question we compared the growth properties of the normal lung cells NHBE and WI-38 to their derivatives expressing *Sdccag1* shRNA, and the lung cancer cell lines A549 and EKVX to their derivatives expressing fly *Clbn*. We first examined their abilities to form colonies when grown on soft agar. While neither NHBE nor NHBE-pSMc21 could form colonies on soft agar, we were surprised to see that WI-38, WI-38-pSMc20 and WI-38-pSMc21 could form colonies (Figure 5a and data not shown). Inhibition of human Sdccag1 had no effect on colony formation.

A549 and EKVX cells form numerous large colonies when grown on soft agar; however, expression of fly Clbn greatly reduced both the number and size of colonies (Figure 5a, b). As expression of Sdccag1 was reported to result in G1 cell cycle arrest (Carbonnelle *et al*., 2001), we initially established cell lines to use the tetracycline repressor to prevent constitutive expression of fly Clbn, but examination of our lines revealed that, first, the repression of fly Clbn by the tetracycline repressor was minimal in all the lines we established (Figure 5b and data not shown) and, second, its expression does not cause cell cycle arrest.

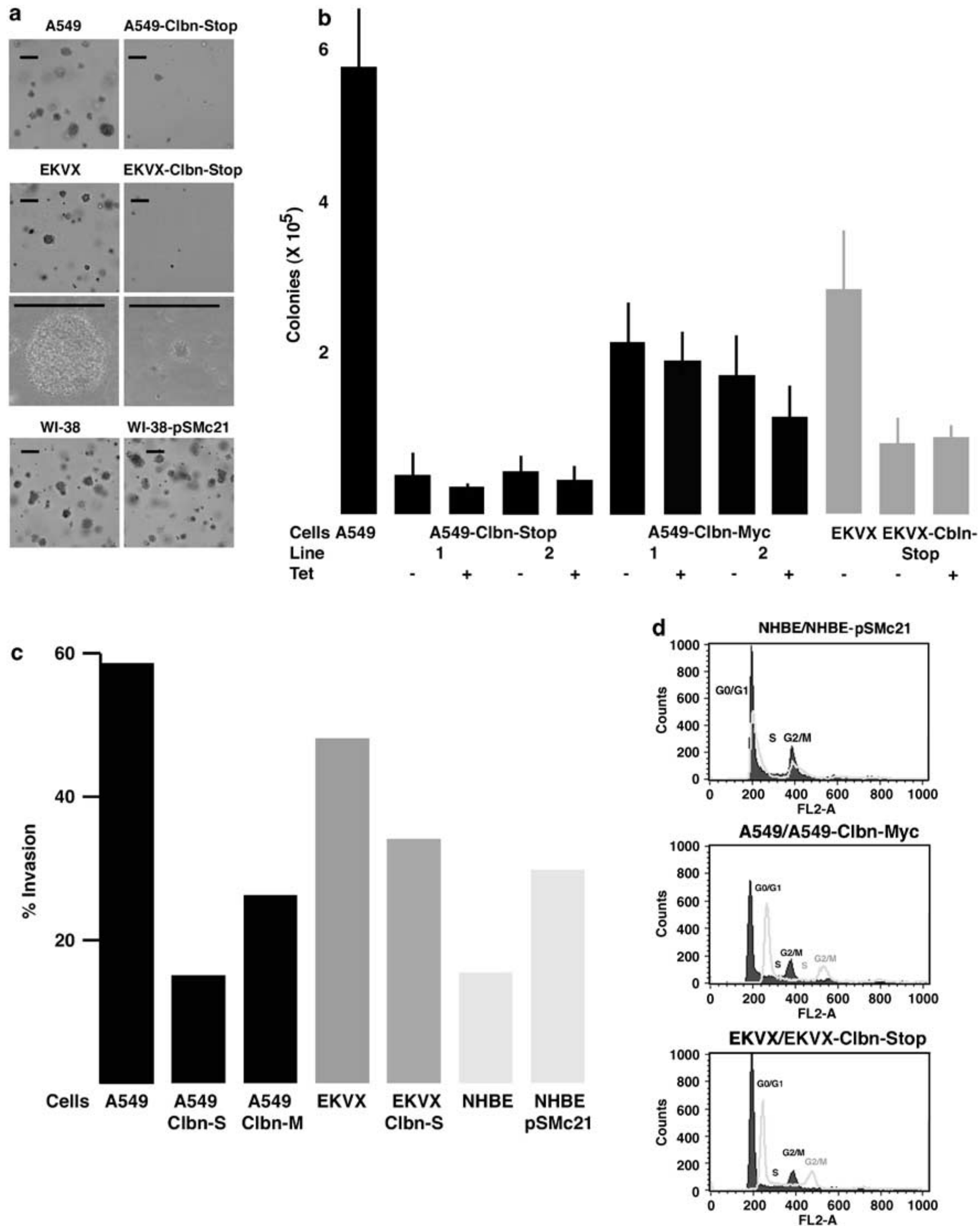

Figure 5 Fly Clbn is a tumor suppressor in human lung cancer cells. (**a, b**) Expression of fly Clbn in A549 or EKVX lung cancer cells impedes their formation of colonies when grown on soft agar. Bars indicate 100 μm. A549, EKVX and the 'noncancerous' lung cell line WI-38 form numerous large colonies when grown on soft agar. Expression of fly Clbn greatly reduces the size **(a)** and number of colonies **(a, b)**. Removal of human Sdccag1 (WI-38-pSMc21) has no effect on colony formation (**a**). (**c**) Expression of fly Clbn reduces the invasiveness of A549 and EKVX cells and inactivation of endogenous Sdccag1 in NHBE cells increases invasiveness. (**d**) Inactivation of Sdccag1 in NHBE cells and expression of fly Clbn in A549 and EKVX cells have only minor effects on the cell cycle

To measure whether Clbn/Sdccag1 expression might play a role in the metastatic progression of cancer, we performed invasion assays. We compared the ability of cells to migrate through 8 μm pores that were uncoated (control) or coated (experimental) with extracellular matrix components (Matrigel, BD Biosciences).

Equal numbers of cells were plated on experimental and control plates and invasiveness was calculated as the quotient of the number of cells passing through the pores of the experimental plate divided by those doing so on the control plate. Cells that can more readily pass through the pores of the experimental plates compared to the control plates have a higher metastatic potential.

We compared invasiveness for the cell lines A549, A549-Clbn-S and A549-Clbn-M. Expression of fly Clbn reduced the percent invasiveness of A549 cells (Figure 5c). A reduction in invasiveness was also observed in EKVX cells that expressed fly Clbn. We next measured invasiveness of the primary cell line NHBE and its derivative, NHBE-pSMc21. Inhibition of Sdccag1 in NHBE cells increased invasiveness (Figure 5c).

Activation of human Sdccag1 had been reported to result in G0/G1 cell cycle arrest in NSCLC-N6 cells (Carbonnelle *et al*., 2001); however, the constitutive expression of fly Clbn neither results in arrest nor changes the length of the cell cycle of A549 (approximately 24 h) and EKVX (approximately 48 h) cells. Furthermore, inhibition of human Sdccag1 with shRNAs also did not appear to have an effect on the cell cycle lengths of NHBE and WI-38 cells. We have noted that while A549 and EKVX cells can survive in media without the addition of serum or growth factors, their derivatives expressing fly Clbn do not survive in the absence of serum.

We performed a FACS analysis in order to more precisely compare the cell cycles of NHBE to NHBE-pSMc21, A549 to A549-Clbn-S and A549-Clbn-M, and EKVX to EKVX-Clbn-S (Figure 5d). Overall there is little difference between the primary cell line NHBE and its derivative with human Sdccag1 inactivated and between A549 and EKVX and their derivatives expressing fly Clbn. We do note small changes in the proportion of cells in G0/G1. For example, we found that 70% of NHBE cells in log growth were in G0/G1; however, this percent increased to 75% in NHBE-pSMc21 cells. In total, 82% of EKVX cells were in G0/G1 while the percentage was only 60% of EKVX-Clbn-S cells. It remains to be determined if these small differences play a role in cellular behavior.

Drosophila *clbn*

The requirement for *clbn* gene expression during *Drosophila* development is unknown and no mutations existed in the gene at the start of this project. As a first step in determining its function, we performed RNA *in situ* hybridization on developing *Drosophila* embryos (Tautz and Pfeifle, 1989). A digoxigene-labeled antisense probe showed that *clbn* is expressed ubiquitously throughout embryonic development (data not shown).

As no *clbn* mutations were extant, we used two methods for creating flies that lacked Clbn function. First, we used homologous recombination to delete the fly *clbn* gene. Second, we created transgenic flies that can express the dominant-negative form of Clbn, Clbn-C (see Figure 3e). The characterization of these flies is ongoing and will not be described in detail here. In brief, homozygous knockout *clbn* flies are viable and fertile. Expression of the dominant negative Clbn-C, either ubiquitously or in the same cells that express Prospero, results in 20% embryonic lethality, with the majority of the remaining flies surviving to adulthood. Examination of Prospero protein in neuroblasts of *clbn* knockout flies did not show a relocalization of the protein to the nucleus (data not shown). At this time any requirement for Prospero nuclear export during normal *Drosophila* development remains unclear.

Discussion

We had previously identified an Exportin-dependent nuclear export signal and a nuclear export signal-masking region in the carboxy terminus of the Prospero protein (Demidenko *et al*., 2001; Bi *et al*., 2003). These domains function in both *Drosophila* and mammalian cells, demonstrating that the regulatory machinery is conserved from flies to man. While Prospero subcellular localization changes dramatically throughout *Drosophila* development (Doe *et al*., 1991; Vaessin *et al*., 1991; Matsuzaki *et al*., 1992), the role played by nuclear export in this process remains unknown. As our next step in determining the role of nuclear export in the regulation of Prospero, we performed a yeast two-hybrid experiment, using the export signal as bait, and identified one protein, Clbn, which was postulated to regulate this process.

Here we demonstrate that fly Clbn and its human homolog, Sdccag1, mediate the nuclear export of fusion proteins containing the HDA NES. When EYFP-HDA is transfected into normal fly or primate cells, or several human cancer cell lines, the fusion protein is exported from the nucleus of many of the transfected cells (Figures 3b and 4a; also see Demidenko *et al*., 2001). When these cells express Clbn/Sdccag1 RNAi, nuclear export is abrogated (Figures 3c, 4c). Thus, Clbn/Sdccag1 activity is essential for the export of HDA fusion proteins. Furthermore, export of EYFP-HDA can be restored in lung cancer cell lines, which do not normally have this export function, by expressing fly Clbn (Figure 4a, d). This suggests that the only activity missing from human lung cancer cells, preventing the export of HDA fusion proteins, is Sdccag1. Other than fly Prospero, we have not yet identified putative *in vivo* targets for Clbn and Sdccag1 regulated nuclear export.

We show that fly Clbn is a bipartite mediator of nuclear export. While the carboxy terminus binds HDA (Figure 1) and presumably other proteins as cargo, the amino-terminus, which has an abundance of conserved leucine residues, is an Exportin-dependent nuclear export signal (Figure 3). We further showed that this region of Clbn binds Exportin *in vitro* (Figure 1c). Thus, fly Clbn most likely regulates nuclear export *in vivo* by directly binding both Exportin and cargo proteins.

Clbn is conserved from yeast to man. Fly Clbn is 49% identical to human Sdccag1 over the entirety of the protein (Figure 2). Conservation is even higher over the Exportin and HDA binding regions, as well as a central region of unknown function. As Sdccag1 is required *in vivo* for export of HDA fusion proteins, the most parsimonious explanation is that it also functions as a bipartite nuclear export mediator.

As Sdccag1 had been implicated in colon and lung cancer, we decided to test (1) whether EYFP-HDA could be used as a biomarker for Sdccag1 activity and if so (2) whether Sdccag1 activity is disrupted in some human cancer cell lines. While EYFP-HDA was exported in two colon and two of three central nervous system cancer cell lines (data not shown), five independent non-small-cell lung cancer lines fail to show export (Figure 4a and data not shown). However, normal human lung cells express *sdccag1*RNA and export EYFP-HDA (Figure 4a, b). Export is Sdccag1 dependent as RNAi, which disrupts its translation, abrogates this function (Figure 4c). Furthermore, expression of fly Clbn in lung cancer cells restores nuclear export (Figure 4d). We conclude that HDA fusion proteins can be used as effective biomarkers for Sdccag1 activity and that non-small-cell lung cancer cells have inactive Sdccag1.

Does inactivation of *sdccag1* play a contributory role in transforming lung cells from normal to malignant? In order to answer this question, we inactivated Sdccag1 function in normal lung cells by stably integrating constructs that produce shRNA containing sequences from the *sdccag1* gene. We also stably integrated constructs that express full-length fly Clbn. We then assayed the malignancy potential for the parent and derivative cell lines.

Inactivation of Sdccag1 in the primary human lung epithelial cell line, NHBE, and the immortalized embryonic lung cell line, WI-38, had little effect on the potential malignancy of the cells. This is not surprising as cancer progression is a multistep process (reviewed in Vogelstein and Kinzler, 2004). In brief, there was little effect on the cell cycle length or the ability to form colonies on soft agar. However, NHBE-pSMc21 cells do appear to be more invasive than their parent cells (Figure 5c). They also showed a minor increase in the percentage of log growth cells that were in G0/G1 (Figure 5d). The significance of these differences is not understood at the moment.

In contrast, expression of fly Clbn demonstrated that it is a potent tumor suppressor in the non-small-cell lung cancer lines examined (Figure 5). Both formation of colonies on soft agar and invasiveness are greatly reduced in A549 and EKVX lung cancer cell lines constitutively expressing fly Clbn (Figure 5a–c). We see, however, only a small effect on the cell cycle. The length of the cell cycle is unaffected and there is only a minor change in the percentage of cells in G0/G1 (Figure 5d). Taken together, our results suggest that fly Clbn and human Sdccag1 are excellent candidates for use in gene therapy for lung and possibly other human cancers.

Materials and methods

Yeast two-hybrid screen

The 64 amino-acid homeodomain of Prospero was cloned into pGBKT7 (BD Biosciences) and used as bait to screen oligo dT primed *Drosophila* pooled 21 h Canton S embryonic mRNA in pACT2 (BD Biosciences). Cotransformed yeast (AH109) were transferred onto SD/-Leu/-His/-Ade/-Trp/X-α-gal/5 mM 3-AT plates (high stringency selection) and incubated at 30°C for 4–6 days. Surviving colonies were restreaked on low stringency SD/-Leu/-Trp/X-α-gal plates2–3 times and reselected on high stringency plates. Plasmid DNA was isolated from surviving yeast and analysed by polymerase chain reaction (PCR) and restriction digestion with *Hae*III. Unique clones and frame shift mutant versions were retransformed into AH109, mated to yeast transformed with pGBKT7-HD and again selected on high stringency plates.

Co-immunoprecipitation

Protein was expressed and labeled with ^{35}S-methionine by *in vitro* transcription and translation using TNT T7-coupled reticulocyte lysate system (Promega) with the vectors pGADT7-Clbn-C, pGBKT7-HD, pGADT7-HD, pGADT7-Clbn, pGBKT7-Clbn-N and pGADT7-hCrm1. pQE-RanQ69L was expressed in BL21 cells and RanQ69LGTP was purified by NTA-resin chromatography and dialyzed in the presence of 0.1 mM GTP. In all, 5 μl of each *in vitro* synthesized product were mixed and incubated 1 h at 30°C, with bacterially expressed RanQ69LGTP added where indicated. Mixtures were then diluted in 470 μl co-IP buffer (20 mM Tris.Hcl pH 7.5, 150 mM NaCl, 1 mM DTT, 5 μl/ml aprotinin, 0.5 mM PMSD, 0.1% Tween 20), 10 μl Protein-A agarose and 10 μlC-Myc antibody, HA antibody (Roche), or anti-Clbn, and then incubated at 4°C 2 h or overnight. Samples were then centrifuged 2 min and washed in TBST (20 mM Tris.Hcl pH 7.5, 150 mM NaCl, 0.1% Tween 20) four times. Pellets were resuspended in 15 μl SDS-loading buffer, heated to 80°C for 5 min, briefly spun down and loaded on 4–12% gradient SDS–PAGE gels. Gels were fixed, washed in H_2O, and NAMP100 was added for 20 min before drying and exposing to film.

RNA hybridization in situ

Whole-mount embryo *in situ* hybridization is described elsewhere (Tautz and Pfeifle, 1989). Digoxigene-labeled sense and antisense probes were transcribed by T7 and SP6 RNA polymerase, respectively, from *Xho*I or *Eco*RI digested pSP73-Clbn using the DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions. Signal was detected by alkaline phosphatase histochemical staining.

Synthesis of double-stranded RNAi

Both strands of linearized templates (pSP73, pSP73-EYFP, pSP73-Clbn, pSP73-Emb, pSP73-hCrm1) were transcribed using T7 and SP6 RNA polymerase (Promega's RiboMax large-scale RNA production system). Double-stranded RNA was then prepared as descrbed elsewhere (Misquitta and

Paterson, 1999), visualized on 1% agarose gels, and quantified by determining the optical density at 260 nm.

Human sdccag1 Northern blot

Reverse transcription–PCR was used to amplify a 309 bp cDNA from the carboxy terminus of Sdccag1 (2978–3286 bps from sequence NM_004713.2). This cDNA was cloned into pPCR-Script-Amp (Stratagene), labeled with ^{32}P and hybridized to a Human Northern Light Blot (Panomics) according to the manufacturer's instructions.

Tissue culture and transfection

Drosophila SL2 cells were grown at 25°C in HyQ CCM3 (HyClone) supplemented with gentomycin sulfate. Cells were split, incubated 2 days to 80–90% confluence and transfected as follows: 6 μl Fugene6 (Roche) was added to 100 μl HyQ media, followed by 2 μg of DNA of the vector pAc5.1/V5-HisA-EYFP-HDA, incubated 20 min and then mixed with the aforementioned cells. Cells were grown for 2–3 days, transferred to fresh media in six-well plates containing poly-lysine (0.1 mg/ml)-coated glass cover slips, incubated 4–6 h, during which time cells attach to the coverslips, and then fixed, stained and examined by confocal microscopy as described elsewhere (Demidenko *et al*., 2001).

Mammalian CV1, WI-38 and IMR-90 cells were purchased from American Type Culture Collection. A primary NHBE was purchased from Cambrex. Cancer cell lines were a gift of S- Amundson and A- Fornace. Mammalian cells were cultured, transfected, fixed and analysed as described elsewhere (Demidenko *et al*., 2001). pEYFP-HDA (Demidenko *et al*., 2001), pEYFP-Pros-NLS+ (Bi *et al*., 2003) and pREV(1.4)-GFP-PKI (Henderson and Eleftheriou, 2000) are described elsewhere.

Creation of stable transformed cell lines

Full length Clbn was cloned into pcDNA/TO/myc-His-C (Invitrogen) with and without the endogenous translation stop sequence, the latter tags the protein with the myc epitope. These were linearized by digestion with *Ssp*I and stable cell lines selected on zeocin (50 μg/ml). pcDNA6/TR (Invitrogen), which expresses the Tet repressor, was linearized by digestion with *Sap*I and stable cell lines selected on blasticidin (1–5 μg/ml). Stable cell lines expressing a short hairpin RNA that functions as *sdccag1* RNAi were generated by stably integrating pSM2c (Open Biosystems) containing either of two short sequences of *sdccag1* (nucleotides 163–185 or 1181–1202 from NM_004713.2) linearized by digestion with *Apa*I and stable cell lines selected with puromycin (25–50 ng/ml).

Soft agar colony assay

Cells were suspended in DMEM containing 0.4% agarose and 1 ml/well was plated in six-well plates (35 mm) over a layer of DMEM containing 0.8% agarose. After hardening, 2 ml/well of DMEM plus 10% FBS was added and the cells were incubated at 37°C in 5% CO_2. Media were replaced every 2–5 days for 3–5 weeks. Colonies were photographed and counted by an inverted phase contrast microscope. Selection drugs for the stably transformed cell lines were added as indicated.

Invasion assay

Invasion chambers (BD Biosciences) were used according to the manufacturer's instructions. In brief, equal numbers of cells in media without serum were placed in experimental (basement membrane coated) and control (uncoated) chambers on membranes with 8 μM pores. These were placed in 6- or 24-well plates containing media with serum. After incubation for 22 h (A549) or 44 h (EKVX and NHBE), cells that moved through the pores were fixed in methanol, stained with Geimsa and counted. Data are presented as percent invasion, calculated by dividing the number of cells from experimental chambers by those in control chambers.

FACS analysis

Cells were grown to 80–90% confluence in T75 flasks, harvested with trypsin, pelleted, resuspended in 70% EtOH at −20°C and stored until needed. Cells were resuspended in PBT at room temperature and stained with propidium iodide (50 μg/ml) in the presence of 0.1 mM EDTA and RNase A + T (50 μg/ml and 0.5 U/ml, respectively) for 1 h. Cells were analysed on a FACScan flow cytometer (Becton Dickson).

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