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G_2 arrest and impaired nucleocytoplasmic transport in mouse embryos lacking the proto-oncogene *CAN/Nup214*

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The vertebrate nucleopore complex (NPC) is a 125 MDa multiprotein assembly that mediates nucleocytoplasmic transport. One of its components, CAN/Nup214, is an FXFG repeat-containing protein known to be involved in myeloid leukemia in humans. We have devised a powerful genetic approach, using maternally derived protein in murine null embryos, to show that CAN/Nup214 is essential for NPC function *in vivo*. We demonstrate that *CAN–/–* mouse embryonic stem (ES) cells are not viable and that *CAN–/–* embryos die *in utero* between 4.0 and 4.5 days postcoitum, following the depletion of their CAN from maternal sources. In 3.5-day-old mutant embryos, cultured *in vitro*, progressive depletion of CAN leads to cell cycle arrest in G_2 phase, and eventually to blastocoel collapse, impaired NLS-mediated protein uptake and nuclear accumulation of polyadenylated RNA. Remarkably, these defective CAN-depleted embryos do not display any gross morphological abnormalities in their nuclear envelopes or NPCs. Our data suggest that CAN is critical to cell cycle progression and required for both nuclear protein import and mRNA export.

Keywords: CAN(Nup214)/cell cycle/gene targeting/nucleocytoplasmic trafficking/oncogenesis

Introduction

The human *CAN* gene was first identified as a target of chromosomal translocation (6;9)(p23;q34) associated with a subtype of acute myeloid leukemia (von Lindern *et al.*, 1992). CAN, also called Nup214, is a nuclear pore complex (NPC) protein: it associates with the NPC and its primary sequence contains multiple copies of NPC protein-specific peptide repeat motifs (Kraemer *et al.*, 1994; Fornerod *et al.*, 1995). Some 100 different proteins (nucleoporins) make up the NPC, a 125 MDa supramolecular structure that crosses the nuclear envelope and mediates bidirectional transport of macromolecules between the cytoplasm and the nucleus (reviewed by Rout and Wente, 1994; see also Panté and Aebi, 1994; Davis, 1995; Doye and Hurt, 1995; Görlich and Mattaj, 1996; Hurt, 1996).

Selective protein import into the nucleus is a two-step process. First, a heterodimeric complex composed of the nuclear localization signal (NLS) receptor and p97 binds to a karyophilic substrate in the cytosol. The NLS receptor of the heterodimer recognizes the substrate's NLS (Adam and Gerace, 1991; Adam and Adam, 1994; Görlich *et al.*, 1994), and p97 may mediate binding of the import complex to FXFG repeat-containing nucleoporins associated with the NPC (Chi *et al.*, 1995; Görlich *et al.*, 1995; Moroianu *et al.*, 1995a; Radu *et al.*, 1995a). Second, the entire complex moves through the central channel of the NPC. This process is energy-dependent and mediated by the small GTPase Ran (Melchior *et al.*, 1993; Moore and Blobel, 1993). The import complex components are then released into the nucleoplasm, except for p97, which is retained at the nuclear side of the NPC (Görlich *et al.*, 1995; Moroianu *et al.*, 1995a). Export of proteins and ribonucleoproteins (RNPs) from the nucleus is also an active, energy-dependent and factor-mediated process, and short protein sequences that act as nuclear export signals (NESs) have recently been identified in some of these factors (Bogerd *et al.*, 1995; Fischer *et al.*, 1995; Fritz *et al.*, 1995; Gerace, 1995; Stutz *et al.*, 1995; Wen *et al.*, 1995; Görlich and Mattaj, 1996).

Over the past few years, a number of NPC proteins have been identified in yeast and biochemical and genetic approaches have shed some light on their roles in nucleocytoplasmic transport (Davis, 1995; Doye and Hurt, 1995; Rexach and Blobel, 1995). Yet, the lack of both accurate protein import assays and experiments that localize the proteins within the NPC, together with pleiotropic defects in some nucleoporin mutants, have hampered precise interpretation of functional studies. In higher eukaryotes, most nucleoporins identified to date have been sublocalized to specific NPC regions (Panté and Aebi, 1994), but their *in vivo* function(s) remain to be explored by genetic tools.

Results

CAN is an essential nucleoporin

As a first step towards genetic dissection of the NPC we disrupted the murine *CAN* gene by using gene targeting and ES cell technology (Figure 1A). Heterozygous mutant mice were interbred and their offspring genotyped by Southern blot analysis (Figure 1B). None of the 233 pups screened was homozygous for the disrupted *CAN* allele, demonstrating that CAN is essential for embryonic development. To determine the time at which the CAN gene disruption is lethal, 3.5-, 6.5-, 7.5- and 9.5-day-old embryos were isolated and their genotypes were determined by polymerase chain reaction (PCR) amplification of DNA fragments diagnostic for the wild-type and the disrupted *CAN* allele (data not shown). No *CAN–/–* embryos were found at days 6.5, 7.5 and 9.5 of gestation; however, at 3.5 days postcoitum, *CAN–/–* embryos were detected at a normal Mendelian frequency of 25%. These homozygous mutant blastocysts were morphologically indistinguishable from their wild-type and heterozygous counterparts.

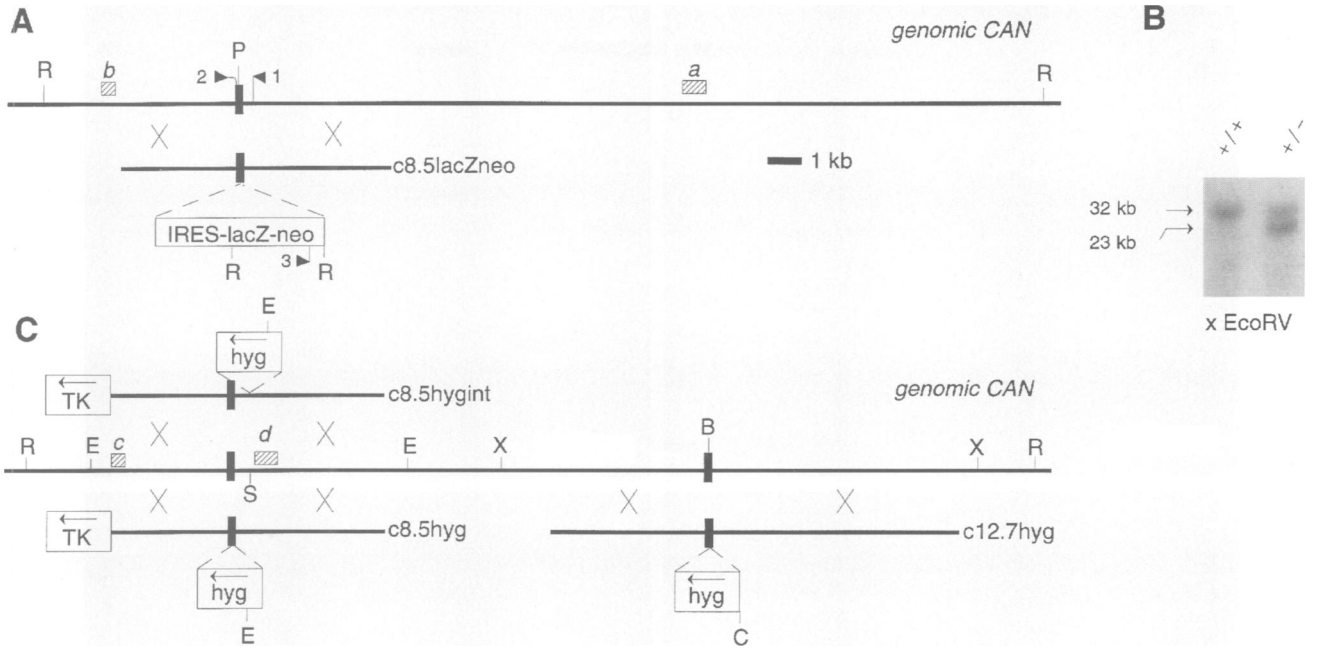

Fig. 1. Targeted disruptions of the mouse *CAN* gene in ES cells. (**A**) Schematic diagram showing a 32 kb *Eco*RV fragment of the mouse *CAN* locus and primary targeting vector c8.5lacZneo. The solid box indicates the mouse exon that corresponds to codons 278–429 of the human *CAN* gene. Shaded boxes mark probes used for Southern blot screening of homologous recombinants and arrowheads indicate the positions of the three primers used to PCR genotype the embryos. (**B**) Southern blot analysis of wild-type (+/+) E14 ES cells and targeted ES clone 65 (+/–). *Eco*RV-digested DNA hybridized to probe *a*. Arrows indicate the positions of the wild-type (32 kb) and lacZneo-disrupted alleles (23 kb). (**C**) Schema of the wild-type *CAN* allele of heterozygous mutant ES cells and the three targeting vectors used in the second rounds of homologous recombination. Restriction sites and probes (*c* and *d*, shaded boxes) used in Southern blot analysis are indicated. Abbreviations: TK, HSV-tk gene; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; P, *Pvu*II; R, *Eco*RV; S, *Stu*I; X, *Xba*I.

To evaluate the role of CAN further, we wanted to inactivate the remaining functional *CAN* allele in heterozygous ES cells. First, *CAN*+/– ES cells were selected in high concentrations of G418 (Mortensen *et al.*, 1992), but none of the 34 resistant clones showed duplication of the mutant *CAN* allele. Second, *CAN*+/– ES cells were subjected to a second round of homologous recombination by using vector c8.5hyg (Figure 1C). Twelve targeted ES clones were obtained; strikingly, all homologous recombination events occurred at the previously disrupted allele. A second double-targeting vector, c12.7hyg (Figure 1C), yielded 105 targeted clones but, again, all recombination events occurred at the previously disrupted *CAN* allele. The above results strongly suggest that inactivation of both *CAN* alleles is incompatible with ES cell survival.

To exclude the possibility that the wild-type allele in the *CAN*+/– ES clones was refractory to homologous recombination, we constructed and tested a third double-targeting vector, c8.5hygint (Figure 1C). This vector was designed to insert a selectable marker in the *CAN* gene without disrupting its coding sequence. Nine of 23 double-targeted *CAN*+/– clones showed recombination at the wild-type allele, thereby indicating that this allele is accessible to homologous recombination, and supporting our conclusion that CAN is essential for cell survival.

How can homozygous null embryos survive during the early steps of development, yet ES cells that lack the CAN protein die? To test whether maternally derived CAN (Kidder, 1992) could account for the normal development of homozygous mutants during very early embryogenesis, we immunostained fertilized eggs (0.5 day postcoitum; $n=35$), 2-cell embryos (1.5 days postcoitum; $n=24$), 8/16-cell embryos (2.5 days postcoitum; $n=39$), blastocysts (3.5 days postcoitum; $n=15$) and hatched blastocysts (4.0 and 4.5 days postcoitum; $n=15$ and $n=39$, respectively) with affinity-purified anti-CAN antibodies (αCNN76-2). We found that the intensity of staining of 0.5-, 1.5- and 2.5-day-old mutant embryos was indistinguishable from that in wild-type and heterozygous embryos (Figure 2A and B). In contrast, cells of *CAN*–/– blastocysts stained very weakly for CAN (Figure 2C and D) and *CAN*–/– day 4.0 hatched blastocysts did not stain at all (Figure 2E and F). At 4.5 days no CAN-negative hatched blastocysts were observed. These results indicate that CAN levels appear normal in mutant embryos up to 2.5 days postcoitum; however, over the next 24 h CAN levels drop and the embryos die between day 4.0 and 4.5 because of the ongoing CAN depletion.

Growth arrest and degradation of cultured CAN–/– embryos

To understand the degeneration of *CAN*–/– embryos better, we isolated blastocysts from matings between heterozygous mutant mice, cultured them, and examined them microscopically. Initially, wild-type, heterozygous and homozygous mutant blastocysts, genotyped by PCR, were morphologically indistinguishable (Figure 3A and B). However, after 18–24 h, the blastocoel of homozygous mutant embryos ($n=22$) gradually became smaller until it had completely collapsed 2–3 h later (Figure 3B–D). Trypan blue staining of these collapsed embryos revealed no signs of cell death. In contrast, 91% of wild-type ($n=23$) and 96% of heterozygous embryos ($n=44$) hatched

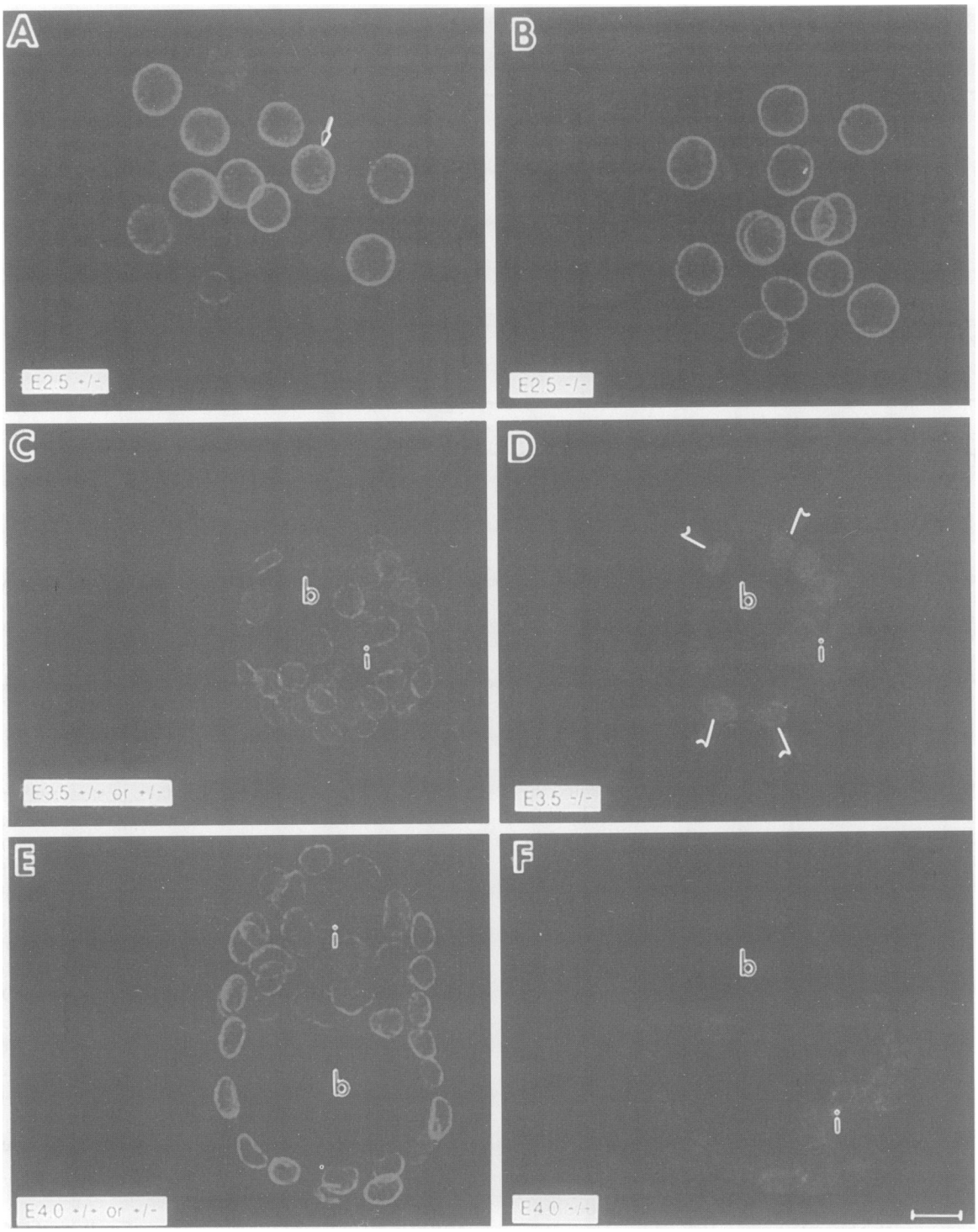

Fig. 2. Indirect immunofluorescence detection of CAN in pre-implantation embryos from *CAN*+/– interbreedings. Representative confocal images of 2.5- to 4-day-old embryos stained with affinity-purified anti-CAN antibodies (αCNN76-2). (**A**) Cells of a heterozygous mutant embryo at day 2.5 showing CAN-specific staining of the nuclear membrane, indicated by the arrow. (**B**) Nuclear envelopes of embryonic cells from a 2.5-day-old homozygous mutant embryo stained with similar intensity as normal embryonic cells. (**C** and **E**) In wild-type or heterozygous embryos (day 3.5 and 4), CAN is localized to the nuclear membrane of inner cell mass cells (labeled i) and of the trophectoderm cells that shape the blastocoel (labeled b). (**D**) In mutant 3.5-day-old embryos, CAN-specific staining is barely detectable; arrowheads point to cells with weak nuclear envelope staining. (**F**) A hatched *CAN*–/– blastocyst, 4 days postcoitum, does not stain for CAN. The scale bar in (F) represents 14 μm.

normally from the zona pellucida and attached to the culture dish during the second day of culture.

Mutant and control blastocysts contained similar numbers of cells at the start of culture [38±10 (n=3) versus 37±5 (n=26)] cells, respectively). In contrast, at the onset of blastocoel contraction, *CAN*–/– embryos contained considerably fewer cells than did controls [45±7 (n=12) versus 76±4 (n=11) cells, respectively], indicating that very few cells of the mutant blastocyst cells had divided in culture.

We then examined whether the cells of *CAN*–/– blastocysts were arrested at a particular stage of the cell

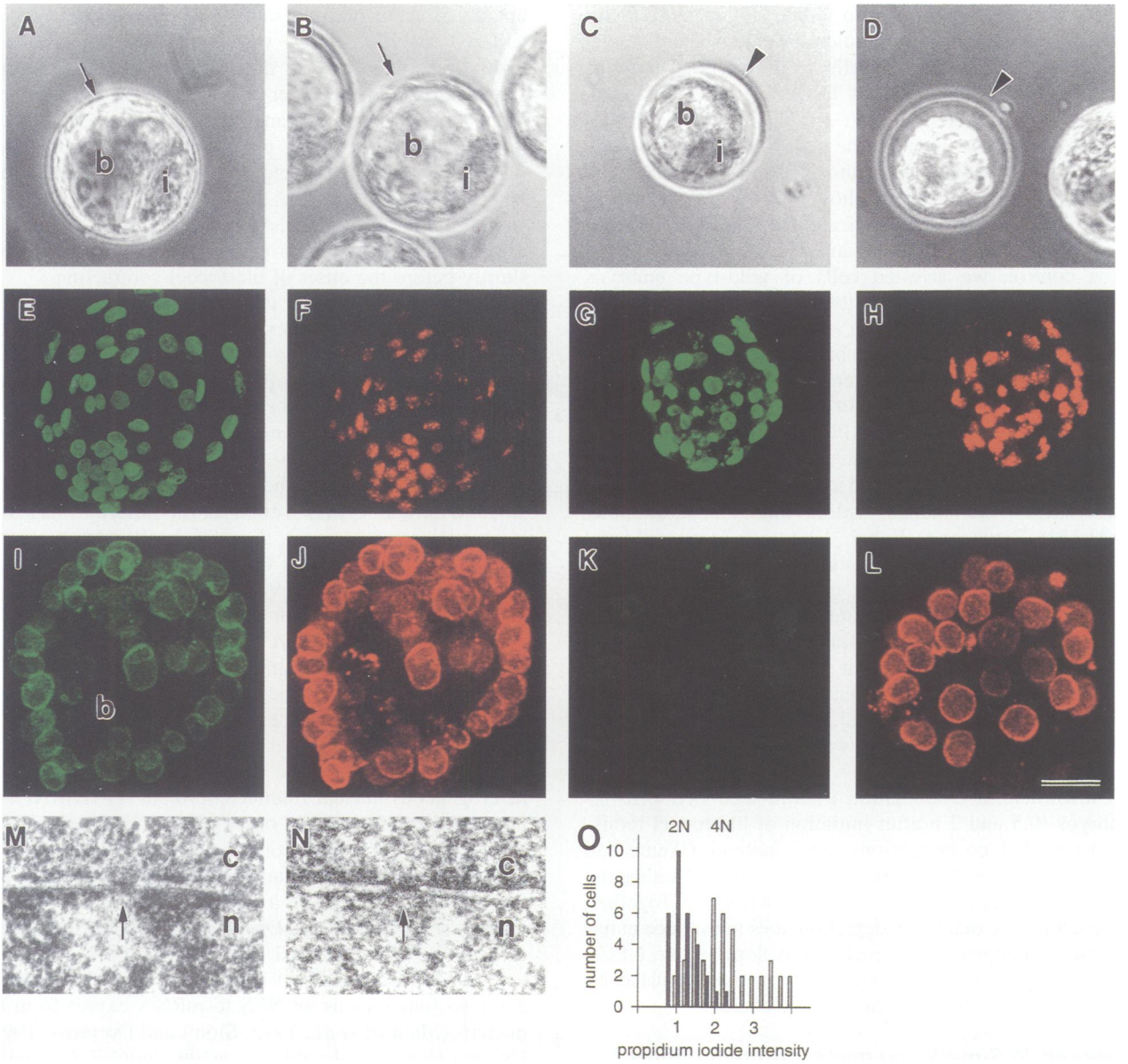

Fig. 3. Growth and morphology of CAN-depleted blastocysts cultured *in vitro*. (**A–D**) Phase contrast images of representative 3.5-day-old embryos cultured *in vitro*. (A) Wild-type embryo cultured for 18 h; the morphology of the embryo remained unchanged over the next 5 h. (B–D) A *CAN–/–* embryo at various stages of *in vitro* culture: (B) at $t = 18$ h (~1 h before the onset of its blastocoel collapse); (C) 2 h later; (D) 4 h later. Arrows indicate trophectoderm cells; b, blastocoel; i, inner cell mass. Arrowheads point to the zona pellucida. (**E–H**) DNA replication and DNA content of blastocysts cultured *in vitro* for 20 h in the presence of BrdU. (E and F) Confocal images of a control blastocyst stained for BrdU (E) and propidium iodide (F). (G and H) A representative *CAN–/–* embryo at the onset of its blastocoel collapse (at ~20 h of *in vitro* culture) stained for BrdU (G) and propidium iodide (H). Note that almost all mutant nuclei stain strongly for propidium iodide. (E and G) and (F and H) were scanned and photographed under identical conditions. (**I–L**) Nuclear envelope morphology and NPC distribution as viewed by immunofluorescence microscopy. (I and J) Confocal images of a control blastocyst (cultured for 24 h) stained with (I) αCNN76-2 and (J) mAb414 (10 μg/ml). (K and L) A representative CAN-depleted embryo 2 h after onset of its blastocoel remission ($t = 26$ h of *in vitro* culture) stained with (K) αCNN76-2 and (L) mAb414. (**M** and **N**) Electron micrographs of the nuclear envelopes of control and degenerating embryos. Detail of (M) a normal and (N) a collapsed blastocyst (~2 h after onset of blastocoel contraction). n, nucleus; c, cytoplasm; arrows indicate the nuclear pores. The bar in (L) represents the length of 30 μm in (A–D), 36 μm in (E–H), 18 μm in (INL), and 155 nm in (M) and (N). (**O**) Histogram of the DNA content of control (black) and mutant embryonic cells (gray) after 20 h of culture derived from cytometric analysis. Propidium iodide fluorescence is indicated in arbitrary units. 2N, unreplicated DNA; 4N fully replicated DNA.

cycle. For this study, 3.5-day-old embryos from *CAN+/–* intercrosses were isolated and cultured in the presence of bromodeoxyuridine (BrdU) until the *CAN–/–* embryos started to collapse (18–24 h). The BrdU incorporated into the DNA during S phase was then assessed by using indirect immunofluorescence with an anti-BrdU antibody. The embryos were also stained for DNA with a fluorescent marker, propidium iodide, to quantitate the total DNA content of their cells by image cytometric measurements. BrdU did not affect growth and development of mouse embryos *in vitro*. All of the nuclei of both control embryos ($n=7$; Figure 3E) and *CAN–/–* embryos ($n=5$ embryos; Figure 3G) stained for BrdU, which demonstrated the ability of *CAN–/–* cells to enter and progress through S phase. In contrast to embryonic nuclei of controls (Figure 3F), the vast majority of nuclei of the mutant embryos

stained strongly for propidium iodide (Figure 3H). Cytometric quantitation of propidium iodide stained nuclei revealed that 75–85% of cells from *CAN–/–* embryos contained a 4N equivalent of DNA. On the other hand, only 10–15% of cells in *CAN+/–* and *CAN+/+* embryos showed a 4N DNA content: the majority of cells from the control embryos undergo mitosis during the 18–24 h culture period and therefore show a DNA content of ~2N (Figure 3O). These results indicate that *CAN–/–* cells seem to progress through S phase and become arrested in G_2. As a control, we arrested cells of wild-type embryos ($n=16$) in G_2 by treating them with Hoechst 33342 (0.15 μg/ml, $n=10$; Tobey *et al.*, 1990), and found that the G_2 arrest does not induce blastocyst degeneration.

To test whether *in vitro* degeneration of murine *CAN–/–* embryos is accompanied by alterations in the morphology of the nuclear envelope or NPCs, as has been shown in yeast (Doye *et al.*, 1994; Wente and Blobel, 1994; Gorsch *et al.*, 1995; Li *et al.*, 1995), embryos from *CAN+/–* intercrosses were stained with a monoclonal antibody (mAb414; Davis and Blobel, 1986), to O-glycosylated nucleoporins. Indirect immunofluorescence showed a punctate nuclear rim staining in all cells of the *CAN–/–* embryos for at least 6 h after the onset of blastocoel remission (Figure 3L). This staining pattern was indistinguishable from that seen in *CAN+/+* and *CAN+/–* embryos (Figure 3J). Moreover, there was no evidence of NPC clustering. We also did not observe fractionated nuclei, indicating that there were no apoptotic cells in these collapsed embryos.

Ultrastructural examination of thin-sectioned *CAN–/–* embryos (0.5 and 2 h after initiation of blastocoel remission) revealed no herniations, invaginations (Wente and Blobel, 1993, 1994), or other abnormalities of either the nuclear envelope or the NPCs (Figure 3M and N). Together, these data show that CAN depletion does not induce major structural changes in the nuclear envelope or the NPCs, and that the death of *CAN–/–* cells is likely caused by functional rather than structural deficiencies.

Impaired NLS-mediated nuclear protein import

We examined whether CAN depletion affects nuclear protein import in embryonic cells by assaying the *in vitro* import of fluorescent allophycocyanin protein (APC) chemically coupled to the SV40 large T NLS peptide (APC–NLS; Adam *et al.*, 1990). We assessed APC–NLS accumulation at the onset of blastocoel contraction in the nuclei of digitonin-permeabilized cells from *CAN–/–* and control embryos after 5 and 10 min incubations at 25°C. As shown in Figure 4A–D, the nuclei of both control and CAN-depleted embryos at the onset of blastocoel contraction accumulated increasing amounts of APC–NLS during the assay period; however, the rate of import was always significantly lower for the mutants ($P<0.05$). The average nuclear concentration of APC–NLS after 5 min incubations was 45–60% lower for the *CAN–/–* embryos ($n=8$; Figure 4B) than for the control embryos ($n=8$; Figure 4A) in the same import reaction. Import of APC–NLS was selective because APC without an NLS did not accumulate in the embryonic nuclei ($n=4$ embryos; Figure 4I). We also analyzed nuclear import at two earlier stages of *in vitro* culture when *CAN–/–* embryos appeared morphologically indistinguishable from controls. Nuclear uptake of APC–NLS in mutant embryos that had been cultured for 12 h ($n=4$) was similar to that in control embryos cultured for the same amount of time (data not shown). In contrast, after about 20 h in culture, four *CAN–/–* embryos (compare Figure 4G and H) showed impaired NLS-mediated protein import (24–33% less APC–NLS imported compared with control embryos), whereas seven other *CAN–/–* embryos still displayed similar import as controls (compare Figure 4E and F). These results show that protein import becomes defective shortly before the onset of blastocoel contraction.

Recent results suggest that p97 may mediate docking of cytosolic import complexes to repeat-containing nucleoporins associated with the NPC (Radu *et al.*, 1995b). To test for abnormalities in p97 binding to CAN-depleted NPCs, we immunostained embryos derived from *CAN+/–* interbreedings with a monoclonal antibody to p97 (mAb3E9; Chi *et al.*, 1995). We observed a strong punctate nuclear rim staining in the cells of the controls (Figure 4J) and of the *CAN–/–* embryos at different stages of blastocoel contraction (1, 3 and 6 h after onset; Figure 4K). It demonstrates that CAN-depleted NPCs can bind p97, suggesting that the p97-mediated docking of nuclear proteins to the NPC is not solely dependent on CAN.

We also examined the *in vivo* localization of the nuclear protein DEK by using immunofluorescence with anti-DEK antibodies (Fornerod *et al.*, 1995). DEK localized to the nucleus of *CAN–/–* embryos stained 1 and 3 h after the onset of the blastocoel collapse (data not shown). In addition, we analyzed the subcellular distribution of the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) in mutant embryos at the onset of blastocoel contraction. hnRNP A1 plays an important role in the biogenesis of mRNA. It shuttles continuously between the nucleus and the cytosol, although it localizes predominantly to the nucleus (Piñol-Roma and Dreyfuss, 1992). Nuclear uptake of hnRNP A1 is mediated by a 38 amino acid sequence, termed M9, that is distinct from classical NLS sequences, and also functions as an NES to mRNA export from the nucleus (Michael *et al.*, 1995; Siomi and Dreyfuss, 1995). Using a monoclonal antibody against hnRNP A1 (9H10) to immunostain *CAN–/–* ($n=5$) and control embryos ($n=7$) at the onset of blastocoel contraction, we found that hnRNP A1 localized properly and did not accumulate in the cytoplasm of *CAN–/–* embryos (Figure 4L and M).

Nuclear accumulation of poly(A)$^+$ RNA

We next examined embryonic cells for nuclear export of mRNA. Subcellular localization of poly(A)$^+$ RNA in mutant embryos was studied at various time points before and after onset of blastocoel contraction by *in situ* hybridization with an oligo(dT)$_{50}$ probe directly coupled to FITC (Figure 5A, C, E and G). For reference in each of the hybridized embryos the position of the nuclei is shown by double staining with propidium iodide (Figure 5B, D, F and H). In the cells of control embryos ($n=12$), poly(A)$^+$ RNA was diffusely distributed in the nucleoplasm and cytoplasm (Figure 5A). By contrast, in *CAN–/–* embryos ($n=14$) that were at the onset of blastocoel contraction or 1 to 4 h thereafter (Figure 5C), we found strong nuclear staining, demonstrating nuclear accumulation of poly(A)$^+$ RNA. As control on the specificity of our mRNA detection method, embryos pretreated with RNase were stained.

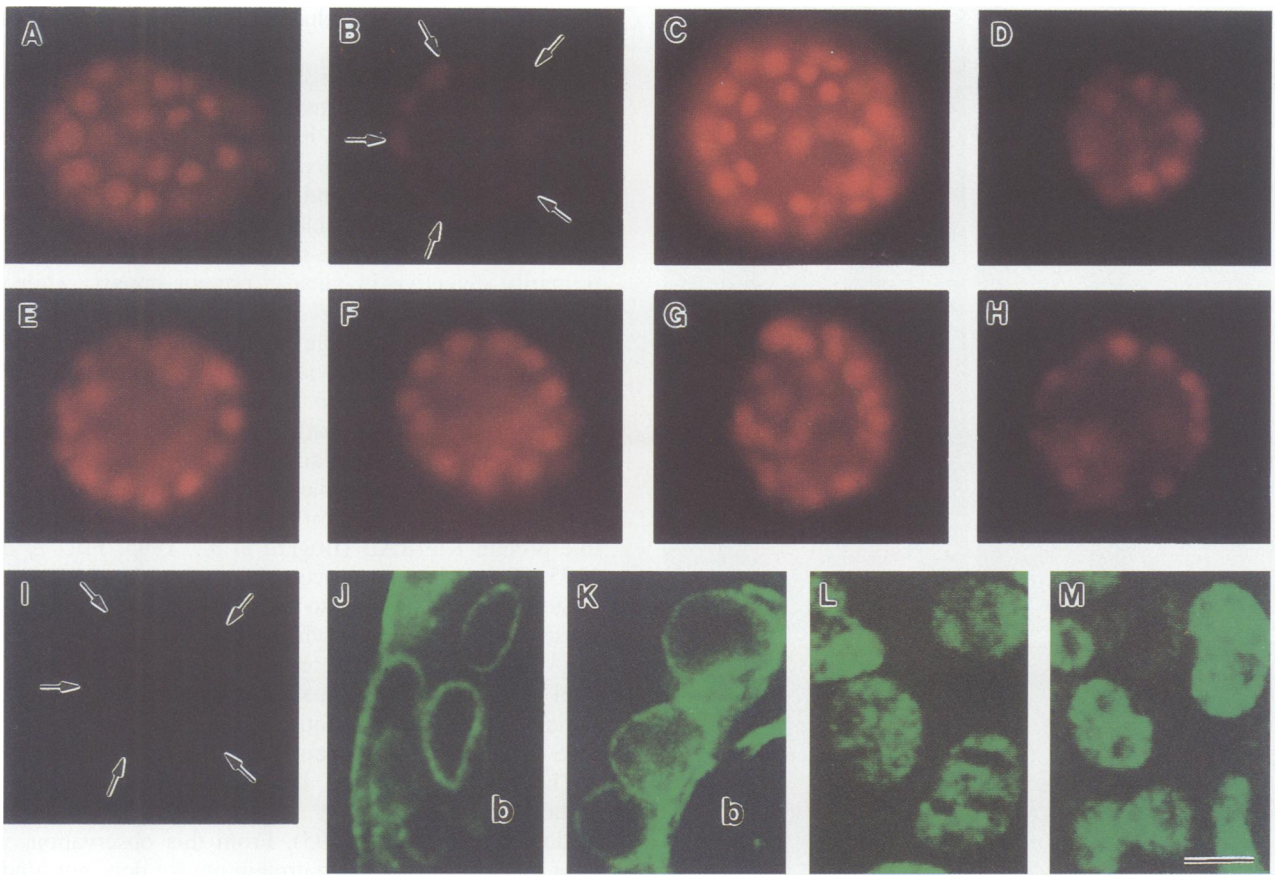

Fig. 4. Nuclear import of karyophilic substrates and subcellular distributions of p97 and hnRNP A1. (**A–H**) Representative nuclear protein import assays on cultured blastocysts. Import of APC–NLS into the nuclei of control (A and C) and *CAN–/–* embryos at the onset of blastocoel collapse (after 20.5 h of culture) (B and D), after 5 min (A and B), and after 10 min (C and D) incubations in transport mixture. All four embryos were from the same import experiment. The average nuclear concentration of APC–NLS was 45% lower in (B) than in (A), and 52% lower in (D) than in (C). Import of APC–NLS into the nuclei of control (E and G) and *CAN–/–* embryos (F and H) cultured for 20 h (but lacking any signs of blastocoel collapse) incubated for 5 min in transport mixture. The average nuclear concentration of APC–NLS was similar in (E) and (F), but was 30% lower in (H) than in (G). (**I**) Representative embryo incubated with APC that lacks an NLS. All embryos were examined with a conventional immunofluorescence microscope and photographed under identical conditions. Not all of the fluorescent nuclei were in the same focal plane. Nuclei in the center of embryos (E), (F) and (H) that were not in focus appeared as background fluorescence. (**J–K**) Confocal microscopy of embryos immunostained with an antibody to p97 (mAb3E9; 2 μg/ml). p97 localized to the nuclear membrane and the cytoplasm (weak staining) of control (J) and *CAN–/–* embryonic cells (K; 1 h after onset of blastocoel remission). (**L–M**) Confocal images of embryos immunostained with a monoclonal antibody to hnRNP A1 (mAb9H10; 1 in 500 dilution). hnRNP A1 predominantly localizes to the nuclei of both control (L) and *CAN–/–* (M) embryos at the onset of blastocoel contraction. Unstained areas represent cytoplasmic compartments of embryonic cells. Abbreviation: b, blastocoel. The outline of embryos (B) and (I) marked by arrows. The scale bar in (M) represents 18 μm in (A–I), and 8 μm in (J–M).

Typically, such embryos showed little or no cytoplasmic or nuclear staining (Figure 5G and H).

We also assayed the mRNA export status at two earlier stages of culture, when mutants and controls were still microscopically indistinguishable. *CAN–/–* embryos (n=4 embryos) grown *in vitro* for 12 h, had normal poly(A)$^+$ RNA distribution patterns (data not shown). Yet, after 20 h of culture, two out of four homozygous mutant embryos had begun to accumulate poly(A)$^+$ RNA (Figure 5E and F). This result shows that the block in RNA export precedes the onset of blastocoel contraction.

Discussion

We have demonstrated that expression of CAN/Nup214, an FXFG repeat-containing component of the NPC, is critical to NLS-mediated nuclear protein import and poly(A)$^+$ RNA export. Radu *et al.* (1995b) have recently shown that repeat-containing nucleoporins, immobilized on nitrocellulose, can bind a model substrate in the presence of the NLS receptor and p97. This led to the hypothesis that bidirectional transport across the vertebrate NPC may require transport substrates to dock and undock at an array of repeat-containing nucleoporins across the NPC (Moroianu *et al.*, 1995a,b; Radu *et al.*, 1995a). Our finding that NLS-mediated nuclear protein import is significantly reduced in CAN-depleted embryos is consistent with, and provides the first *in vivo* support for this hypothesis. The residual nuclear protein uptake in CAN-depleted embryonic nuclei may reflect some functional compensation by other repeat-containing proteins that are associated with the NPC. Alternatively, it may result from trace amounts of CAN in the NPCs.

Only very few cells of the *CAN–/–* blastocysts divide during the 18–24 h of *in vitro* culture. BrdU labeling experiments and DNA content analysis suggest that these cells are arrested in G_2 phase of their cell cycle. Interestingly, cells expressing *srp1-31*, a temperature-sensitive mutant of the *Saccharomyces cerevisiae* NLS receptor, are also defective in nuclear protein import and arrest in

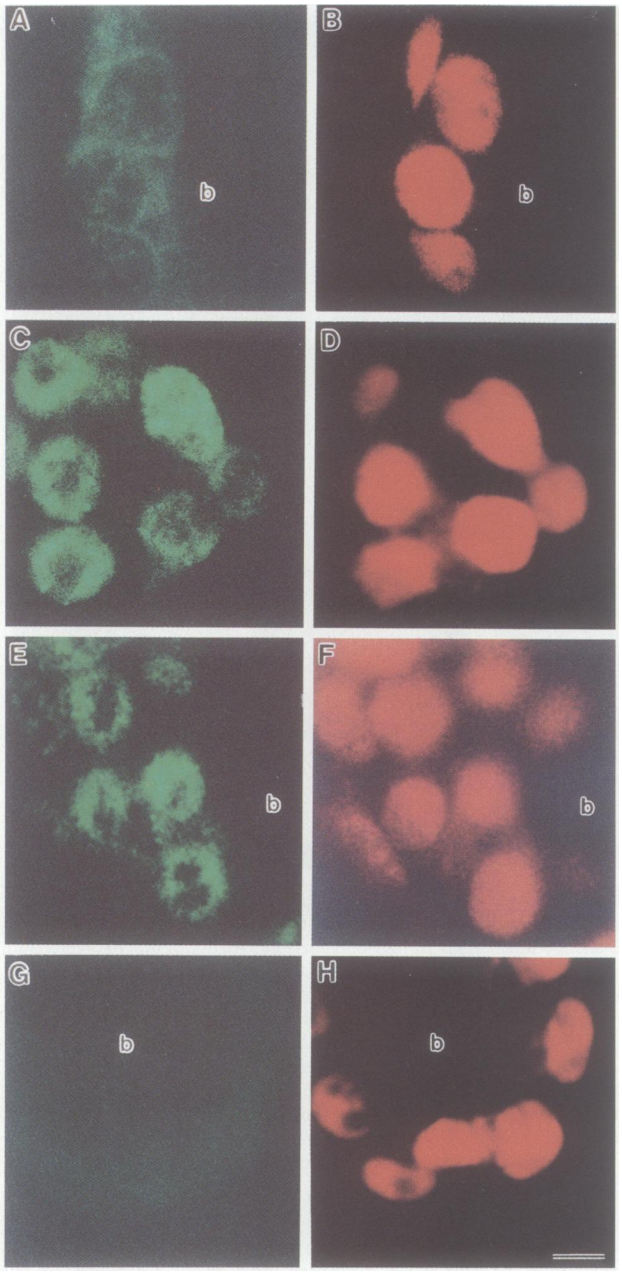

Fig. 5. CAN depletion causes poly(A)$^+$ RNA accumulation in embryonic nuclei. (**A**, **C**, **E** and **G**) Subcellular localization of polyadenylated RNA in embryonic cells analyzed by *in situ* hybridization with an FITC-labeled oligo(dT)$_{50}$ probe. (**B**, **D**, **F** and **H**) Coincident DNA staining with propidium iodide. (A and B) Wild-type embryo (in culture for 24 h); (C and D) embryo homozygous for the disrupted CAN allele, ~2 h after onset of its blastocoel remission (in culture for 24 h); (E and F) *CAN–/–* embryo showing no signs of degeneration after 20 h in culture; (G and H) wild-type embryo pretreated with RNase. Cells from this embryo displayed little nuclear or cytoplasmic staining. Blastocoels are denoted by b. The bar in (H) represents 7 μm.

the cell cycle during the G_2/M phase when cultured under non-permissive conditions (Loeb *et al.*, 1995). The G_2/M arrest in *srp1-31* cells presumably results from a block in import of nuclear protein(s) that mediate a cell cycle-regulated wave of proteolysis that is known to trigger progression through M phase (Amon *et al.*, 1994; Loeb *et al.*, 1995). It is conceivable that the G_2 arrest in the *CAN–/–* embryos is also induced by sensitivity of the cell cycle machinery to reduced nuclear protein import capacity.

CAN–/– embryos not only have impaired NLS-mediated protein import, but their nuclei also accumulate poly(A)$^+$ RNA. These two defects in nucleocytoplasmic transport coincided as CAN was depleted, and may reflect a dual role in protein import and mRNA export, as has been reported for the yeast nucleoporin *NUP49* (Doye *et al.*, 1994). Some temperature-sensitive *NUP49* mutants are primarily defective in nuclear protein import, while in others the initial transport defect occurs at the level of mRNA export. A dual role for CAN in both import and export could be achieved via its FXFG repeats. The FXFG repeats may serve *in vivo* as a docking site for nuclear import of proteins carrying a classical NLS, and they could also have some function in RNA export. This is supported by the remarkable homology between the FXFG repeats of CAN and those of the novel human nucleoporin-like protein RIP/RAB (Bogerd *et al.*, 1995; Fritz *et al.*, 1995). The FXFG repeat-containing domain of RIP/RAB is sufficient for binding to the NES of HIV-1 Rev in a yeast two-hybrid assay, which suggests that the repeats may function as an NES receptor for selective export of viral mRNAs from the nucleus (Gerace, 1995). By the same token, the FXFG motif of CAN may function as an NES receptor in RNA export.

The *srp1-31* mutation blocks NLS-mediated import without causing poly(A)$^+$ RNA accumulation in the nucleus (Loeb *et al.*, 1995). From this observation, we infer that a basic defect in protein uptake does not hinder mRNA export. It supports our contention that the block in mRNA export may be caused by a second function of CAN that is independent from its role in NLS-mediated protein uptake. Like a number of other nucleoporins (Siniossoglou *et al.*, 1996), CAN could be part of one or more subcomplexes within the NPC, and its depletion may simultaneously lead to dissociation of one or more additional nucleoporins from the NPC, for example one that functions directly in mRNA export or protein import.

Just as proteins are selectively imported into the nucleus, RNA export must be carefully controlled. RNP particles move from the site of transcription and assembly to the NPC, where they are translocated to the cytoplasm (Izaurralde and Mattaj, 1995). Nuclear accumulation of poly(A)$^+$ RNA could, therefore, be an indirect consequence of decreased nuclear import of proteins critical to mRNA export that do not employ the classical NLS-mediated pathway for their transport into the nucleus. A prime candidate for such a protein would be hnRNP A1 (Michael *et al.*, 1995). We found no mislocalization of this protein in the cytoplasm of *CAN–/–* cells at a stage when poly(A)$^+$ RNA was retained in the nucleus. This result suggests that the export defect cannot be attributed to the consequences of impaired hnRNP A1 import. It remains to be seen whether this observation holds true for other proteins involved in the poly(A)$^+$ RNA export pathway. The apparently normal nuclear uptake of hnRNP A1 by cells lacking CAN suggests that the M9-mediated nuclear import pathway is independent of CAN.

To conclude, CAN/Nup214 is the first nucleoporin gene to be disrupted in higher eukaryotes to study its function in nucleocytoplasmic transport *in vivo*. We have shown that the presence of maternal gene products in early

embryogenesis permits the use of gene knockout strategies to study the function of genes that are essential for cell survival.

Materials and methods

Targeted disruption of the CAN locus in ES cells

Vector c8.5lacZneo contained an 8.5 kb *Bam*HI–*Sal*I CAN 129Sv/E genomic fragment with an IRES-lacZneo selection cassette (Mountford and Smith, 1995) inserted into a unique *Pvu*II site that corresponds to codon 297 of the human *CAN* gene. Vector c8.5hyg consisted of the same 8.5 kb genomic *CAN* fragment. A hyg^r marker was positioned in its *Pvu*II site and a herpes simplex virus thymidine kinase (HSV-tk) gene cassette was included for negative selection (van Deursen and Wieringa, 1992). Vector c8.5hygint is identical to c8.5hyg except that the hyg^r marker was inserted into an intronic *Stu*I site. Vector c12.7hyg consisted of a 12.7 kb *Hin*dIII *CAN* fragment (from a 129Sv/E genomic library) with a hyg^r marker inserted into a unique *Bam*HI site that corresponds to codon 804 of the human *CAN* gene. These targeting vectors were linearized and electroporated into wild-type or *CAN+/–* E14 mouse ES cells, drug-resistant ES lines isolated, and targeted clones identified for correct replacement events by Southern blot analysis using external probes on *Eco*RV- (c8.5lacZneo; probe a), *Eco*RI- (c8.5hyg and c8.5 hygint; probe c) or *Eco*RV/*Cla*I-cut genomic DNA (c12.7hyg; probe d) as previously described (van Deursen and Wieringa, 1992). Mutant mice were generated as previously described (van Deursen *et al.*, 1993).

Isolation, culture and genotype analysis of mouse embryos

Heterozygous mutant mice, kept on a 6 a.m. to 6 p.m. light–dark schedule, were mated for timed pregnancies. The vaginal plug was scored at 12 p.m. which we designated 0.5 day of development (0.5 day postcoitum). At various times of development, we collected embryos from the uterine horns of plugged *CAN+/–* female mice as described by Hogan *et al.* (1994). Blastocysts, were cultured *in vitro* as described by Hsu (1979).

Embryos were genotyped by PCR amplification. Individual embryos were transferred to Eppendorf tubes containing 1 μl sterile water, to which 3 μl lysis buffer [0.05% SDS (w/v) and 0.035 N NaOH] was added. The samples were boiled for 3 min, and 1.5 μl of this mixture was used for PCR. Embryos at day 6.5, 7.5 and 9.5 were washed extensively in PBS before being transferred to 50 μl of PCR protK buffer [60 mM Tris–HCl, pH 9.0, 15 mM $[NH_4]_2SO_4$, 2 mM $MgCl_2$, 0.5% Tween-20 (v/v) and 250 μg/ml proteinase K]. After incubation for 5 h at 56°C, the proteinase K was inactivated by boiling for 10 min, and 3 μl from each sample was used for PCR. PCR cocktails containing primers (see below) that were diagnostic for the wild-type and lacZneo-disrupted allele were added to the embryo lysates [final concentrations, 60 mM Tris–HCl, pH 9.0, 15 mM $[NH_4]_2SO_4$, 2 mM $MgCl_2$, 200 μM dNTPs, 250 ng of each primer, 180 ng of TaqStart antibody (Clonetech), and 0.8 unit Taq DNA polymerase (Perkin-Elmer Cetus)]. Samples were amplified for 30–35 cycles (94°C for 1 min, 55°C for 2 min, 72°C for 2 min), and PCR products were resolved by electrophoresis through 1.5% agarose gels. The identity of the PCR fragments was confirmed by Southern blot analysis, using an internal CAN oligonucleotide (5′-CACTCCTCGATGTAGCTGAGG-3′) as a probe.

Sequences specific for the wild-type allele were amplified by using, anti-sense (5′-TGGAATCACTCACTGTGGTTG-3′; Figure 1A, primer 1) and sense (5′-GTGAACTTCATGGAGCCCTG-3′; Figure 1A, primer 2) primers that flank the *Pvu*II site within the targeted *CAN* exon (Figure 1A). The targeted allele sequences were amplified by using the same anti-sense *CAN* primer in combination with a sense strand primer for the neo^r gene (5′-TCGTGCTTTACGGTATCGC-3′; Figure 1A, primer 3). These PCR fragments measure 430 bp (wild-type) and 860 bp (lacZ-disrupted), respectively.

Antibody purification and indirect immunofluorescence

Polyclonal rabbit anti-CAN antibodies directed against the N-terminal part of human CAN (αCNN) were affinity-purified by adsorption for 18 h at 4°C to a bacterially produced polypeptide representing amino acids 363–804 of human CAN (Fornerod *et al.*, 1995) bound to Immobulon PVDF membrane (Millipore Corp., Bedford, MA). The membrane was washed five times with PBS containing 0.05% Tween-20 (v/v), and then washed once with PBS alone. Bound antibodies were eluted for 3 min at 0°C in a buffer containing 100 mM glycine at pH 2.5 and 0.05% (w/v) bovine serum albumin. They were then immediately neutralized with 0.05 volumes of 1 M $NaPO_4$ at pH 7.5. The purified antibodies are designated αCNN76-2.

Indirect immunofluorescence was as previously described (Fornerod *et al.*, 1995). Antibody dilutions were 1 in 16 for αCNN76-2, 10 μg/ml for protein A-purified mAb414 (BAbCO), 2 μg/ml for mAb3E9 (Chi *et al.*, 1995), 1 in 1000 for αDEK (Fornerod *et al.*, 1995), and 1 in 500 for 9H10 (anti-hnRNP A1). Primary antibodies were visualized with FITC- or Texas red-conjugated goat anti-mouse or goat anti-rabbit antibodies (10 μg/ml) diluted in PBS containing 1% non-fat dry milk. Embryos were examined by confocal laser scanning microscopy on a Bio-Rad MRC1000.

Electron microscopy

Mutant and control embryos were isolated from microdrop cultures and immediately fixed in 0.1 M phosphate buffer, pH 7.4, containing 2% glutaraldehyde for at least 12 h at 4°C. Embryo samples were then post-fixed in 1% osmium tetroxide for 1 h, dehydrated, and embedded in Spurr low-viscosity resin. Thin sections were cut and examined with a JEOL JEM-1200EX II electron microscope.

BrdU incorporation in cultured blastocysts and DNA content analysis

Embryos were cultured in the presence of 20 μM BrdU for 18–24 h, and then fixed with methanol:acetone (1:1) for 10 min at room temperature (Pagano *et al.*, 1994). After three washes with PBS, embryos were treated with 1.5 N HCl for 10 min at room temperature. After four washes with PBS, embryos were incubated with PBS containing 1% non-fat dry milk for 15 min, and then with anti-BrdU antibodies (undiluted; Amersham, Life Science) for 1 h at room temperature. Anti-BrdU antibody binding was detected with FITC-conjugated goat anti-mouse antibodies (1 in 50 dilution; Amersham, Life Science). Embryos were stained for DNA in PBS containing 100 μg/ml propidium iodide for 60 min at room temperature. After three washes in PBS, embryos were embedded in Vectashield mounting medium, and examined by confocal laser scanning microscopy. The DNA content of individual cells of the scanned embryos was determined by cytometric quantitation of free-lying embryonic nuclei.

Import assay on whole mount embryos

The *in vitro* import assay was done essentially as described (Adam *et al.*, 1990). In brief, two (or occasionally four) embryos cultured in DMEM were rinsed once in transport buffer (20 mM HEPES, pH 7.5, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol and 1 μg/ml of aprotinin, leupeptin, and pepstatin), followed by permeabilization in 80 μg/ml digitonin (Calbiochem; diluted from a 20 mg/ml stock solution in DMSO) in transport buffer on ice for 10 min. After one rinse in transport buffer, the embryos were transferred to 5 μl complete transport mixture which contained 40% reticulocyte lysate, 250 nM APC–NLS (Adam *et al.*, 1990) or unmodified APC as negative control (Calbiochem, San Diego, CA), 1 mM ATP, 10 mM creatine phosphate (Calbiochem), and 20 units/ml creatine phosphokinase (Calbiochem) in transport buffer. Import reactions were carried out in the dark for 5 or 10 min in a humidified chamber at room temperature and terminated by rinsing briefly in transport buffer. The embryos were then immediately examined by epifluorescence (Olympus BX50 microscope) and photographs were taken with a Kodak Royal Gold 1000 film. All embryos were photographed under identical conditions (exposures of 10 s). The concentration of APC–NLS in embryonic nuclei was quantitated by scanning the photographs with a densitometer (X-Rite 810) and deriving the values from a standard curve. On average, 12–18 free-lying nuclei were scanned per embryo. We determined the average nuclear concentration of APC–NLS of each *CAN–/–* and control embryo within the same reaction and compared them. Average nuclear concentrations of APC–NLS of two control embryos within the same import reaction were not significantly different ($n=10$ reactions; $P<0.05$).

In situ hybridization on whole mount embryos

In vitro cultured blastocysts were collected and washed with PBS. The embryos were then fixed in 4% paraformaldehyde (in PBS) for 15 min at 4°C, washed three times in PBS/0.1% Tween-20 (v/v; PBT), and permeabilized in RIPA buffer [50 mM Tris–HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40 (v/v), 0.5% deoxycholate (w/v), 0.1% SDS (w/v)]. After three washes with PBT and one wash with 2× SSC, the embryos were hybridized with an oligo(dT) probe directly coupled to FITC as described (Amberg *et al.*, 1992).

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