

Identification of essential genes in cultured mammalian cells using small interfering RNAs

Jens Harborth^{1,*}, Sayda M. Elbashir^{2,*}, Kim Bechert¹, Thomas Tuschl^{2,‡} and Klaus Weber^{1,‡}

¹Department of Biochemistry and Cell Biology and ²Department of Cellular Biochemistry, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany

*These authors contributed equally to this work

‡Authors for correspondence (e-mail: ttuschl@mpibpc.gwdg.de; office.weber@mpibpc.gwdg.de)

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SUMMARY

We report the first RNAi-induced phenotypes in mammalian cultured cells using RNA interference mediated by duplexes of 21-nt RNAs. The 21 gene products studied have different functions and subcellular localizations. Knockdown experiments monitored by immunofluorescence and immunoblotting show that even major cellular proteins such as actin and vimentin can be silenced efficiently. Genes were classified as essential or nonessential depending on impaired cell growth after RNA silencing. Phenotypes also involved altered cell morphology and aberrant mitotic arrest. Among the essential genes identified by RNAi for which such information was previously not available are lamin B1, lamin B2, NUP153, GAS41, ARC21, cytoplasmic dynein, the protein kinase cdk1 and both β - and γ -actin. Newly defined nonessential genes are emerlin and zyxin. Several genes previously

characterized by other methods such as knockout of murine genes are included as internal controls and gave identical results when RNAi was used. In the case of two nonessential genes (lamin A/C and zyxin) RNAi provides a recognizable phenotype.

Our results complete the characterization of the mammalian nuclear lamins. While lamins A/C appear as nonessential proteins in the mouse embryo and in RNAi treated cultured cells, the two other lamins, B1 and B2, are now identified as essential proteins. Interestingly the inner nuclear membrane protein emerlin, thought to be a ligand of lamin A/C, is also a nonessential protein in tissue culture cells.

Key words: Functional genomics, Gene silencing, Mammalian cells, Nuclear lamins, RNA interference

INTRODUCTION

Gene function has been determined traditionally by methods such as deletion of murine genes, the introduction of (mutated and/or dominant negative) transgenes, the molecular characterization of human hereditary diseases, and targeting of genes by antisense techniques (Porter, 1998). Microinjection of an antibody into tissue culture cells (Blangy et al., 1995) and the use of *Xenopus* oocyte extracts (Desai et al., 1999; Abrieu et al., 2000) can also provide information on protein functions. As the sequencing of the human and murine genomes approaches completion there is an increasing demand for quick, robust and efficient mechanisms to analyse gene function in mammalian cell culture.

RNA interference (RNAi) provides a new approach for elucidation of gene function. RNAi is a sequence-specific, post-transcriptional gene silencing mechanism initiated in animals and plants by the introduction of double stranded RNA (dsRNA) homologous in sequence to the silenced gene (Fire, 1999; Bass, 2000; Cogoni and Macino, 2000; Plasterk and Ketting, 2000; Sijen and Kooter, 2000; Hammond et al., 2001; Matzke et al., 2001; Sharp, 2001; Tuschl, 2001; Waterhouse et al., 2001). RNAi has significantly advanced our understanding of gene function in the nematode *C. elegans* and about one third of the nematode genome has already been subjected to

2000; Barstead, 2001; Hope, 2001; Kim, 2001; Maeda et al., 2001). Mammalian cells were until recently not amenable to RNAi since use of in vitro transcribed, long dsRNAs (>30 bp) led to activation of a global, sequence unspecific response resulting in blockage of initiation of protein synthesis and mRNA degradation (Bass, 2001). Although RNA interference seems to work in early mouse development (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000), it seemed not generally applicable to mammalian cells (Caplen et al., 2000; Ui-Tei et al., 2000).

We recently reported that duplexes of 21-nt RNAs with 2-nt 3' overhang, introduced by transfection into human and other mammalian cultured cells specifically interfered with gene expression and bypassed the sequence independent response of mammalian cells to long dsRNA (Elbashir et al., 2001a). These short RNA duplexes resemble the processing products from long dsRNAs, and are referred to as small interfering RNAs (siRNAs) (Elbashir et al., 2001b). One of the enzymes involved in processing of long dsRNAs has recently been identified and is a member of the RNase III family of nucleases (Bernstein et al., 2001).

We previously documented siRNA-induced silencing of lamin A/C in HeLa cells and mentioned that this approach also worked for lamin B1 and the nuclear mitotic apparatus protein NuMA but not for vimentin. We speculated that the negative

protein or the particular RNA duplex used (Elbashir et al., 2001a). Here we extend our analysis to a large number of genes in mammalian tissue culture cells. We show that RNAi results in silencing of major cellular proteins such as actin and that vimentin can be effectively silenced by selecting three new RNA duplexes. We report the first phenotypes obtained by RNAi in mammalian cells. Among the essential genes identified are lamin B1, lamin B2, NUP153, GAS41, ARC21, cytoplasmic dynein and the mitotic protein kinase cdk1, for which such information was not available previously by ablation of murine genes or other approaches.

MATERIALS AND METHODS

siRNA preparation

To design target-specific siRNA duplexes, we selected sequences of the type AA(N₁₉)UU (N, any nucleotide) from the open reading frame of the targeted mRNA, in order to obtain a 21-nt sense and 21-nt antisense strand with symmetric 2-nt 3' overhangs of identical sequence. We used 2'-deoxythymidines instead of uridine residues in the 3' overhangs to reduce costs of RNA synthesis and to enhance nuclease resistance. It may also be possible to design siRNAs with other sequences in the overhang, or to select siRNAs from the sequences AA(N₂₁) by changing the last two nucleotides of the sense siRNA to TT (the 2-nt overhang of the sense siRNAs is not believed to contribute to target recognition). A selected siRNA sequence was also submitted to a BLAST search against the human genome sequence to ensure that only one gene of the human genome was targeted. 21-nt RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Hamburg, Germany), deprotected and gel-purified as described (Elbashir et al., 2001a; Elbashir et al., 2001b) or purchased from Dharmacon (Lafayette, CO) in deprotected and desalted form.

The accession numbers given below in brackets are from GenBank. The siRNA sequence targeting NuMA (Z11583) was from position 3988-4010 relative to the start codon; GAS41 (NM_006530) 327-349; SV40 T-antigen (62000) 26-48, 639-661; lamin A/C (NM_005572) 608-630; lamin B1 (NM_005573) 672-694; lamin B2 (M94362) 1457-1479 (of the sequence with missing 5'end); LAP2 (NM_003276) 37-59, common region; emerlin (NM_000117) 628-650; Nup153 (NM_005124) 2593-2615; β -actin (NM_001101) 972-994; γ -actin (NM_001614) 8-30; ARC21 (AF006086) 181-203; mouse zyxin (X99063) 1357-1379; mouse vinculin (L18880) 2923-2945; VASP (XM_009141) 1087-1109; vimentin (NM_003380) 346-368, 1145-1167, 863-885, 1037-1059; keratin 18 (NM_000224) 1154-1176; Eg5 (NM_004523) 1547-1569; CENP-E (NM_001813) 944-966; cytoplasmic dynein 1 heavy chain (U53530) 509-531 of partial cDNA; cdk1 (NM_001786) 125-147. As unspecific siRNA control a sequence targeting firefly (*Photinus pyralis*) luciferase gene (X65324) 153-175 was used. siRNA duplex formation (annealing) was performed as previously described (Elbashir et al., 2001a).

Cell culture and transfection

Human HeLa SS6 cells and F5 and FR(wt648) rat fibroblast cells (Zerrahn and Deppert, 1993) were grown at 37°C in Dulbecco's modified Eagle medium supplemented with 10% FCS, penicillin and streptomycin. Cells were regularly passaged to maintain exponential growth. The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics and transferred to 24-well plates (500 μ l per well). Transient transfection of siRNAs was carried out using Oligofectamine (Life Technologies). 12 μ l OPTIMEM 1 medium (Life Technologies) and 3 μ l Oligofectamine per well were preincubated for 5-10 minutes at room temperature. During the time for this incubation 50 μ l OPTIMEM 1 medium were mixed with 3 μ l

minutes at room temperature for complex formation. After addition of 32 μ l of OPTIMEM 1 medium to the mixture, the entire mixture was added to the cells in one well resulting in a final concentration of 100 nM for the siRNAs. The addition of 32 μ l OPTIMEM 1 medium is optional and was only used to adjust the final culture volume to 600 μ l. Cells were usually assayed 40-48 hours after transfection, but in some cases also after 70 hours. Specific silencing was confirmed by at least three independent experiments.

Immunofluorescence microscopy, antibodies and immunoblotting

Immunofluorescence and chromatin staining was performed as described (Elbashir et al., 2001a). Pictures were taken using a Zeiss Axiophot with a F Fluor 40 \times /1.30 oil objective and MetaMorph Imaging Software (Universal Imaging Corporation, West Chester, PA) with equal exposure times for the silenced and the control treated cells. For phase-contrast microscopy, cells were mounted in Hepes buffered DMEM medium supplemented with 10% FCS. Pictures were taken using a Plan-Neofluar 25 \times /0.8 objective.

Several antibodies were kindly provided by B. Burke (monoclonal Nup153), W. Deppert (monoclonal SV40 T-antigen 108), M. Osborn (monoclonal lamin A/C clone 636, NuMA, vimentin monoclonal V9, keratin 18 CK2) and J. Wehland (monoclonal ARC21 7H3, VASP 273D4, vinculin, zyxin 164ID4). Commercial antibodies were from Abcam (β -actin), Novocastra (lamin B2 clone LN43.2, emerlin clone 4G5), Santa Cruz Biotechnology (lamin B1 C-20), Sigma (α -tubulin) and Transduction Laboratories (LAP2 clone 27). The antibody for GAS41 was described previously (Harborth et al., 2000).

For western blotting, transfected cells grown in 24-well plates, were trypsinized, washed once in ice-cold PBS and harvested. Cells from one well were solubilized in 50 μ l SDS sample buffer and boiled for 5 minutes. Equal amounts of total protein were separated on 7.5 or 12.5% polyacrylamide gels and transferred to nitrocellulose. Immunostaining with specific antibodies and peroxidase-conjugated secondary antibodies (Dako, Denmark) diluted 1:20,000 was carried out using the ECL technique (Amersham Pharmacia Biotech). To confirm equal loading, blots were stripped (Re-Blot Western Blot Recycling Kit, Chemicon) and re probed with the vimentin V9 antibody or in case of vimentin silencing with the β -actin antibody.

RESULTS

Selection of target genes

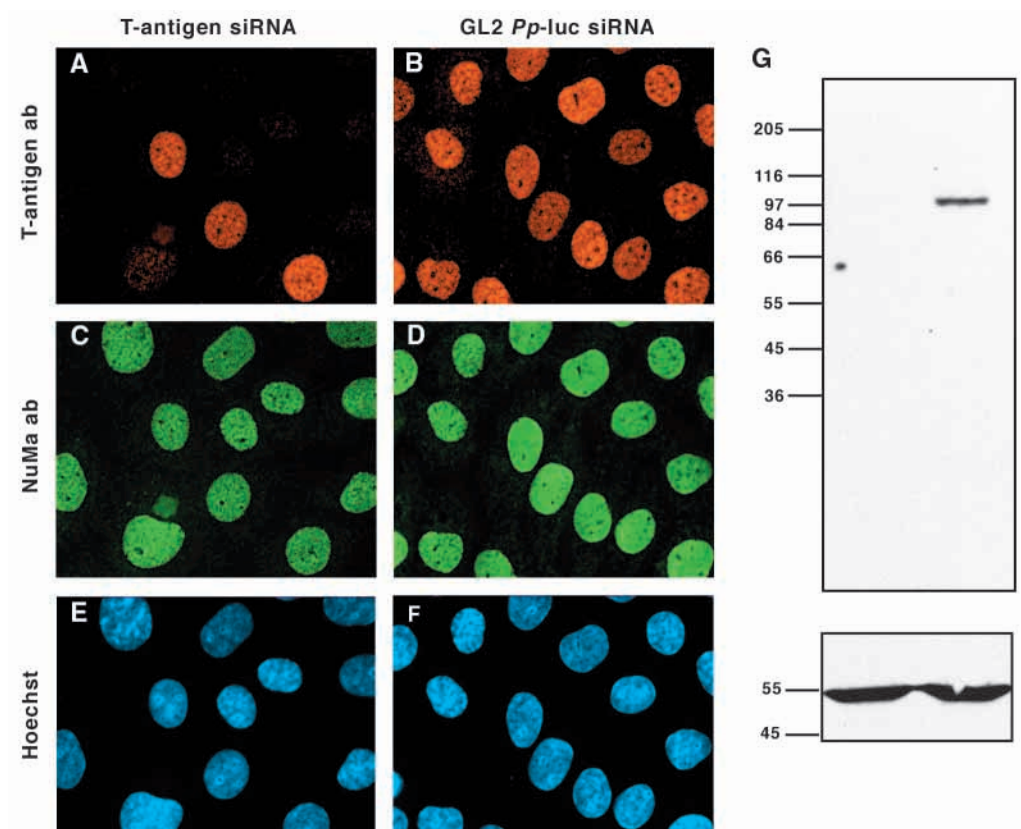
We examined silencing of 21 genes expressed in cultured mammalian cells by RNA interference with duplexes of 21-nt RNAs (Table 1). These included 18 genes in human HeLa cells, 1 gene in SV40-transformed rat fibroblasts, and 2 genes in mouse 3T3 cells. The sequences of the siRNA duplexes were selected from the coding region of the target mRNAs as described in Materials and Methods. In all transfection experiments cultures were transfected in parallel with target-specific siRNA duplexes and a nonspecific duplex of firefly luciferase from *Photinus pyralis* (GL2) at the same final concentration as the specific siRNAs tested (Elbashir et al., 2001a). As a further control we used the addition of buffer instead of the volume of siRNA added to the culture. Gene silencing was documented by immunoblotting and immunofluorescence, except in five cases where no suitable antibodies were available. To screen for phenotypes, we looked for impaired cell growth and altered cell morphology monitored by phase microscopy. Aberrant mitotic arrest was visualized using the α -tubulin antibody.

Table 1. Gene silencing by RNAi: growth arrest and phenotypes in cell culture

Gene	Silencing*	Essential	Phenotypic change	Gene function by other methods
Nuclear proteins				
NuMA	+	+	Apoptotic	Antibody injection, mutant cDNAs: essential for mitosis and nuclear formation
GAS41	+	+	Arrest	
SV40 T-antigen	+	-		
Nuclear envelope proteins				
Lamin A/C	+	-	Emerin displaced	Null mice: nonessential in development; essential after birth
Lamin B1	+	+	Apoptotic	
Lamin B2	+	+	Apoptotic	
LAP2	+	-		
Emerin	+	-		
Nup153	+	+		
Cytoskeletal cytoplasmic proteins				
β Actin	+	+	Blebbing	
γ Actin	nd	+	Blebbing	
ARC21	+	+		
VASP	+	-		
Vinculin	+	+		Null mice: embryonic lethal
Zyxin	+	-	Loss of stress fibers	
Vimentin	+	-		Null mice: nonessential
Keratin 18	+	-		Null mice: nonessential
Mitotic proteins				
Eg5	nd	+	Aberrant mitotic, monopolar spindles	Antibody injection, inhibitor: essential for centrosome separation
CENP-E	nd	+	Aberrant mitotic, multipolar spindles	Antisense and immunodepletion: essential kinetochore-associated motor
Cytoplasmic dynein	nd	+	Aberrant mitotic arrest	
Cdk1	nd	+	Premitotic arrest	

*Gene silencing was monitored by immunofluorescence and/or immunoblotting; nd, not determined because of lack of a suitable antibody. Silencing was performed in human HeLa cells, except for SV40 T antigen (rat fibroblasts) and vinculin and zyxin (mouse 3T3). A stop in growth was taken to indicate that the protein is essential. For gene function by other methods see text.

Fig. 1. Silencing of SV40 large T antigen in a stably transformed rat fibroblast cell line (F5). Triple fluorescence staining of cells transfected with T-antigen siRNA duplex (A,C,E), and with GL2 luciferase siRNA duplex serving as control (B,D,F). (A,B) Staining with T-antigen specific antibody, (C,D) staining with NuMA specific antibody, (E,F) Hoechst staining of chromatin. The few cells in A that are stained for T antigen were probably nontransfected. (G) Western blot of cells transfected with T antigen siRNA (left) or luciferase siRNA (right) probed with T antigen-specific antibody. The blot was stripped and reprobbed with vimentin antibody (bottom).



proteins distributed in different cellular compartments. The study includes three nuclear proteins, six proteins of the nuclear envelope, eight cytoplasmic cytoskeletal proteins, three microtubular motor proteins and the protein kinase cdk1. Seven genes, which were already characterized by other methods such as murine knockouts, microinjection of antibodies and transfection by mutant cDNAs served as controls. In these cases, RNAi mediated by siRNAs provided the same result as the earlier studies (Table 1).

Knockdown of nuclear proteins

Antibody microinjection and transfection with mutant cDNAs showed that the nuclear mitotic apparatus protein NuMA is essential for normal spindles and reformation of nuclei and that NuMA is involved in the early stages of apoptosis (Gueth-Hallonnet et al., 1999). Consistent with these results we observed siRNA-induced growth arrest and detected apoptotic cells (Table 1). NuMA was shown to interact with GAS41, a poorly characterized, but highly conserved protein in eukaryotic nuclei (Harborth et al., 2000). Targeting of GAS41 causes growth arrest of HeLa cells, demonstrating for the first time that GAS41 is an essential protein.

As another example of a nuclear protein we tried to target SV40 large T antigen in transformed rat fibroblasts using two duplexes. Interestingly only one of them resulted in specific silencing (see also vimentin) as demonstrated by immunofluorescence and western blotting (Fig. 1). At least under the conditions used cells continued to grow. It remains to be seen whether this is due to the residual amount of antigen still present or whether T antigen is indeed nonessential for growth.

Knockdown of proteins of the nuclear envelope

The nuclear envelope consists of a double membrane, pores, and the lamina, a filamentous meshwork located between the inner nuclear membrane and the peripheral chromatin. We silenced the nuclear lamins A/C, B1 and B2, the lamina-associated protein LAP2, the inner nuclear membrane protein emerin, and the nuclear pore component Nup153. Knockdown of lamin A/C in HeLa cells did not affect cell growth (Elbashir et al., 2001a), consistent

Fig. 3. Silencing of lamin B1 and lamin B2. HeLa cells were transfected with lamin B1 siRNA (A,C), lamin B2 siRNA (E) or with luciferase siRNA (B,D,F). Cells were either double stained with lamin B1-specific antibody (A,B) and NuMA specific antibody (C,D) or stained for lamin B2 (E,F). (G) Western blot of cells transfected with lamin B1 (left) or luciferase (right) siRNA duplexes using lamin B1 antibody (top). The blot was stripped and re-probed with vimentin antibody to check for equal loading

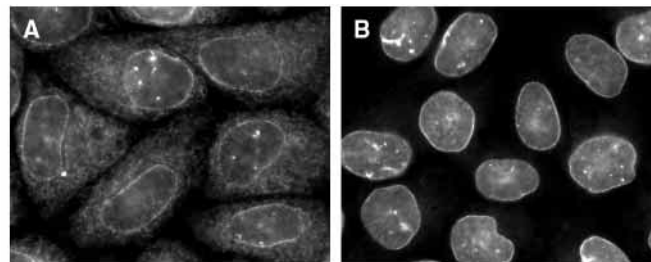
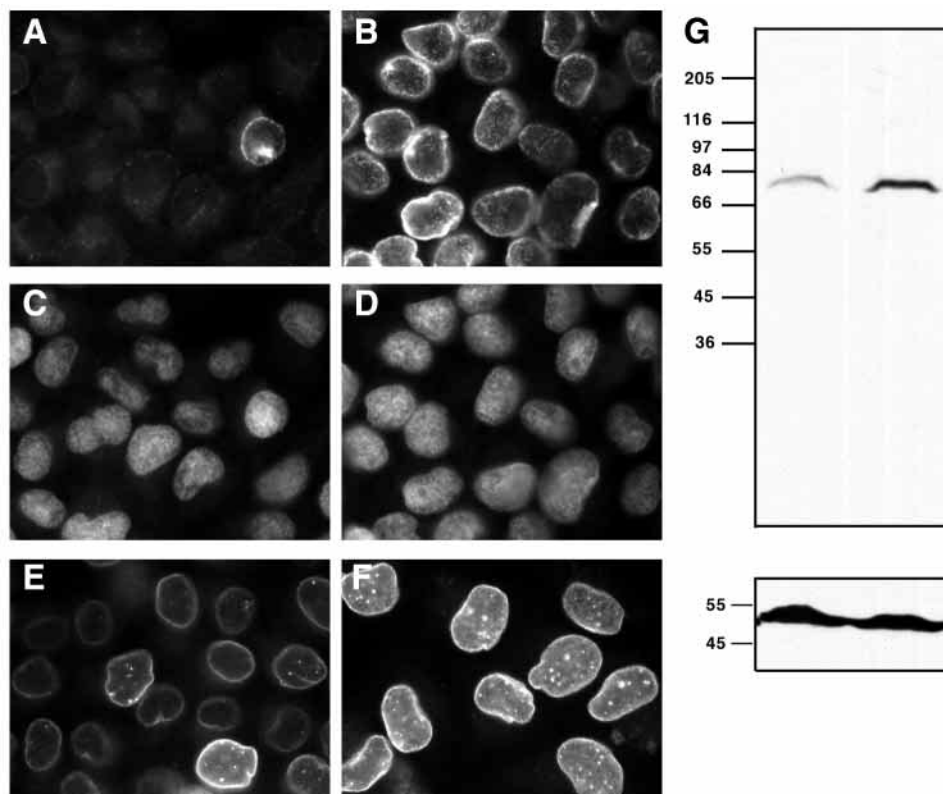


Fig. 2. Silencing of lamin A/C in HeLa cells results in displacement of emerin. Cells transfected with lamin A/C siRNA (A) and with luciferase siRNA (B) were stained with emerin antibody. Cells with reduced lamin A/C expression (A) show enrichment of emerin in the cytoplasm. Magnification 480 \times .

with the observation that mice null for A/C show normal development and only die one to two months after birth due to muscular dystrophy (Sullivan et al., 1999). Also, lamin A/C is acquired only late in development (Röber et al., 1989). Despite the absence of a growth defect, lamin A/C knockdown cells show a distinct phenotype. Similar to fibroblasts derived from embryos of lamin A/C null mice (Sullivan et al., 1999), lamin A/C silenced HeLa cells show an unusual distribution of the inner nuclear membrane protein emerin (Fig. 2). The cytoplasmic distribution of emerin probably within the endoplasmic reticulum is strongly enhanced and supports the view that lamin A/C may serve to immobilize emerin. Therefore, knockdown of genes that are not required for cell growth may still cause distinct phenotypes in cultured cells, which can be monitored by appropriate means.

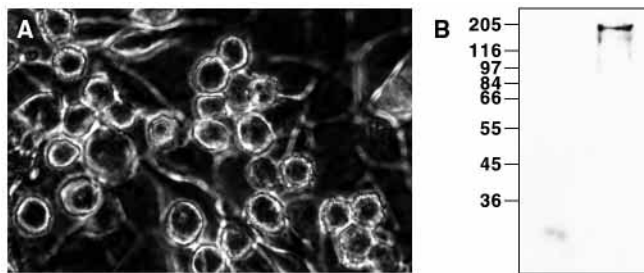


Fig. 4. Silencing of the nuclear pore complex protein Nup153. (A) Phase-contrast of HeLa cells 3 days after transfection with Nup153 siRNA. (B) Western blot of cells transfected with Nup153 siRNA (left) or with luciferase siRNA (right) probed with Nup153-specific antibody. Magnification 300 \times (A).

In contrast to lamin A/C, whose expression is developmentally regulated, lamin B1 and lamin B2 are constitutively expressed (Röber et al., 1989). Lamin B1 and lamin B2 were efficiently silenced 40 hours post-transfection as shown by immunofluorescence or immunoblot (Fig. 3). Later, cells stop growing and become apoptotic. We demonstrated by siRNA-mediated silencing that both B lamins are essential proteins (Table 1). The observed cessation of cell growth upon siRNA transfection indicates that both B-type lamins are necessary to establish a normal nuclear lamina in HeLa cells. Apoptosis induced by lamin B1 and B2 silencing is consistent with the previous finding that perturbation of post-mitotic lamin B1 assembly leads to apoptosis (Burke, 2001). Interestingly transfection with a lamin B1 cDNA carrying a large deletion in the central rod domain leads to lobulated nuclei and growth arrest (Schirmer et al., 2001). These observations do not rule out additional functions for B-type lamins in DNA replication and transcription (Wilson et al., 2001).

The siRNA duplex directed against the nuclear envelope protein LAP2 had no effect on cell growth, yet an effect may have been expected because of the known interaction with the essential B-type lamins (Burke, 2001; Hutchison et al., 2001). Although the LAP2 protein levels were significantly reduced (Table 1), it is unclear whether the residual protein may be responsible for the absence of an effect. Similarly, silencing of the inner nuclear membrane protein emerin, had no effect in HeLa cells. Although lamin A/C knockdown affected emerin localization (see above) (Sullivan et al., 1999), the reverse effect was not observed, and silencing of emerin did not influence the nuclear lamin A/C staining pattern.

Nup153 is a protein of the nuclear pore complex and belongs to the F/GXFG family of nuclear pore proteins (Radu et al., 1995). HeLa cells transfected with siRNAs directed against Nup153 rounded up and showed growth arrest (Fig. 4), indicating that this nuclear pore protein is essential.

Knockdown of cytoskeletal proteins

We previously reported a problem with silencing of vimentin in HeLa cells (Elbashir et al., 2001a) and speculated that this was either due to the abundance of the protein or perhaps due to the sequence selected for siRNA synthesis. We first addressed the issue of abundance and tested siRNAs directed against other major proteins expressed in HeLa cells. Silencing of β -cytoplasmic actin was readily achieved as monitored by a

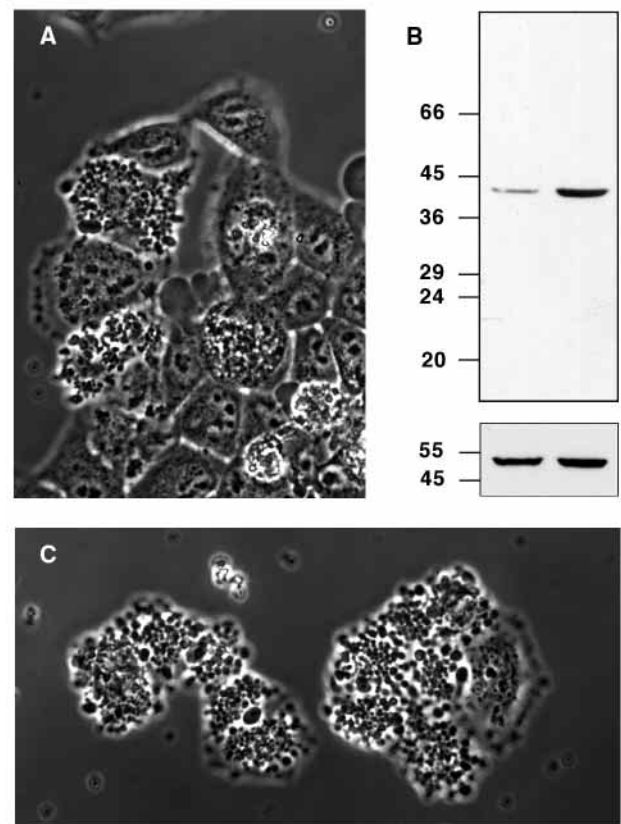


Fig. 5. Silencing of cytoplasmic actins in HeLa cells. (A) Phase-contrast of cells transfected with β -actin siRNA duplex. Transfected cells show blebbing. (B) Western blot of cells transfected with β -actin (left) or luciferase (right) siRNA duplexes using β -actin antibody. The blot was stripped and re-probed with vimentin antibody to check for equal loading. (C) Phase-contrast of cells transfected with γ -actin siRNA duplex. Transfected cells show a blebbing phenotype similar to cells with β -actin knockdown. Magnification 480 \times (A,C).

stop in cell growth and phase microscopy revealed very strong cellular blebbing. The same phenotype was observed after targeting of γ -cytoplasmic actin, although the precise γ -actin-levels could not be measured due to a lack of γ -specific antibody. These results demonstrate that the expression of major cellular proteins can indeed be suppressed by RNAi. Therefore, we tested again for silencing of vimentin with three newly synthesized duplexes directed against different regions of the vimentin mRNA. All newly selected siRNA duplexes were effective in silencing vimentin expression as monitored by immunoblotting. Seventy hours post-transfection, few filaments, which were often fragmented, were detectable by immunofluorescence microscopy (Fig. 6). Some cells contained only short and thin structures, similar to the vimentin squiggles frequently visible in live cells expressing GFP-tagged vimentin (Yoon et al., 1998). The absence of a characteristic phenotype after silencing was expected because vimentin null mice develop and reproduce normally (Colucci-Guyon et al., 1994). Knockdown cells for another intermediate filament protein, keratin 18, behaved similar to vimentin knockdown cells, and keratin 18 knockout mice also appear

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