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Light controllable siRNAs regulate gene suppression and phenotypes in cells

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Abstract

Small interfering RNA (siRNA) is widely recognized as a powerful tool for targeted gene silencing. However, siRNA gene silencing occurs during transfection, limiting its use is in kinetic studies, deciphering toxic and off-target effects and phenotypic assays requiring temporal, and/or spatial regulation. We developed a novel controllable siRNA (csiRNA) that is activated by light. A single photo removable group is coupled during oligonucleotide synthesis to the 5′ end of the antisense strand of the siRNA, which blocks the siRNA's activity. A low dose of light activates the siRNA, independent of transfection resulting in knock down of specific target mRNAs and proteins (GAPDH, p53, survivin, hNuf2) without stimulating non-specific effects such as regulated protein kinase PKR and induction of the interferon response. We demonstrate survivin and hNuf2 csiRNAs temporally knockdown their mRNAs causing multinucleation and cell death by mitotic arrest, respectively. Furthermore, we demonstrate a dose-dependent light regulation of hNuf2 csiRNA activity and resulting phenotype. The light controllable siRNAs are introduced into cells using commercially available reagents including the MPG peptide based delivery system. The csiRNAs are comparable to standard siRNAs in their transfection efficiency and potency of gene silencing. This technology should be of interest for phenotypic assays such as cell survival, cell cycle regulation, and cell development.

Keywords: Light controllable siRNAs to regulate gene expression; MPG delivery for difficult to transfect cell types; Branch DNA quantitation of mRNA expression; Non-specific effects of the double-stranded RNA (dsRNA) regulated protein kinase PKR kinase and induction of the IFN response; Immunofluorescence detection of protein expression; Western blot detection of protein expression; Apoptosis and multinucleation phenotypic assays

1. Introduction

RNA-mediated interference (RNAi) is an evolutionarily conserved mechanism to silence genes [1–4]. Basically, there are two approaches to gene silencing using RNAi: (1) Chemically synthesized double stranded RNA (21–27 nt) known as "small interfering RNA" (siRNA) introduced into cells as active molecules [1–4] and (2) Hairpin precursors "shRNAs" (~25–29 nt) transcribed endogenously and processed within the cell to active siRNAs (~21 nt) [5,6]. In the cell, the siRNA assembles to the protein complex known as "RNA-induced silencing complex" (RISC) which binds to the complementary mRNA via base pairing interactions to the 5′ end of the siRNA antisense strand resulting in a sequence-specific degradation of the mRNA and gene silencing [7]. Both siRNAs and shRNAs have been successfully applied for "loss-of-function" assays with resulting phenotypes in cultured cells and in vivo models [4,8,9]. Although RNAi is a powerful approach for efficiently silencing genes, it does have its limitations since constitutive expression of shRNAs or delivery of active siRNAs cannot address phenotypic assays that require temporal and/ or spatial regulation [10].

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2. Results and discussion

Previously, it was demonstrated that a 5′ end phosphate on the antisense strand is required for a siRNA to function [11]. We have exploited this property by incorporating a single NPE (nitrophenyl ethyl) photo removable "caging" group at the 5′ end of 21-mer siRNA antisense strand as the last step of a conventional nucleotide synthesis (Fig. 1a). Exposure of the csiRNA to light (365 nm), removes the caging group creating an active siRNA. We demonstrated this principle using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) csiRNA (Fig. 1b, c). Increased light energy removed more caging groups until the totally "uncaged" csiRNA electrophoresed to the same position in the gel as the siRNA (Fig. 1b). When the same csiRNA was transfected into HeLa cells and exposed to increasing light energy, a dose-dependent knockdown of target mRNA was demonstrated (Fig. 1c). The amount energy required to fully uncage the GAPDH csiRNA for gel electrophoresis (Fig. 1b) was between 2 and 4 J/cm2 and in cells 1.4 J/cm2 (Fig. 1c). More energy is required to uncage in vitro than in cells because the concentration of csiRNA is significantly higher (Fig. 1b, 5 μM vs. Fig. 1c, 3 nM) and in a vastly larger volume (microliters, Fig. 1b vs. picoliters, Fig. 1c). The difference in energy required agrees with results previously reported by Lin et al. to uncage β-ecdysone in solution and in cells [12].

Highly purified csiRNA antisense strands are important for successful controllable knockdown experiments. We investigated the correlation between the purity of csiRNA and the activity of csiRNA in cells, measured by HPLC chromatograms of the antisense strand. Different lots GAPDH csiRNA of various purities were transfected into HeLa cells and incubated for 24 h at 37 °C. The cells were not exposed to light in order to keep the csiRNA caged and unreactive. The cells were lysed

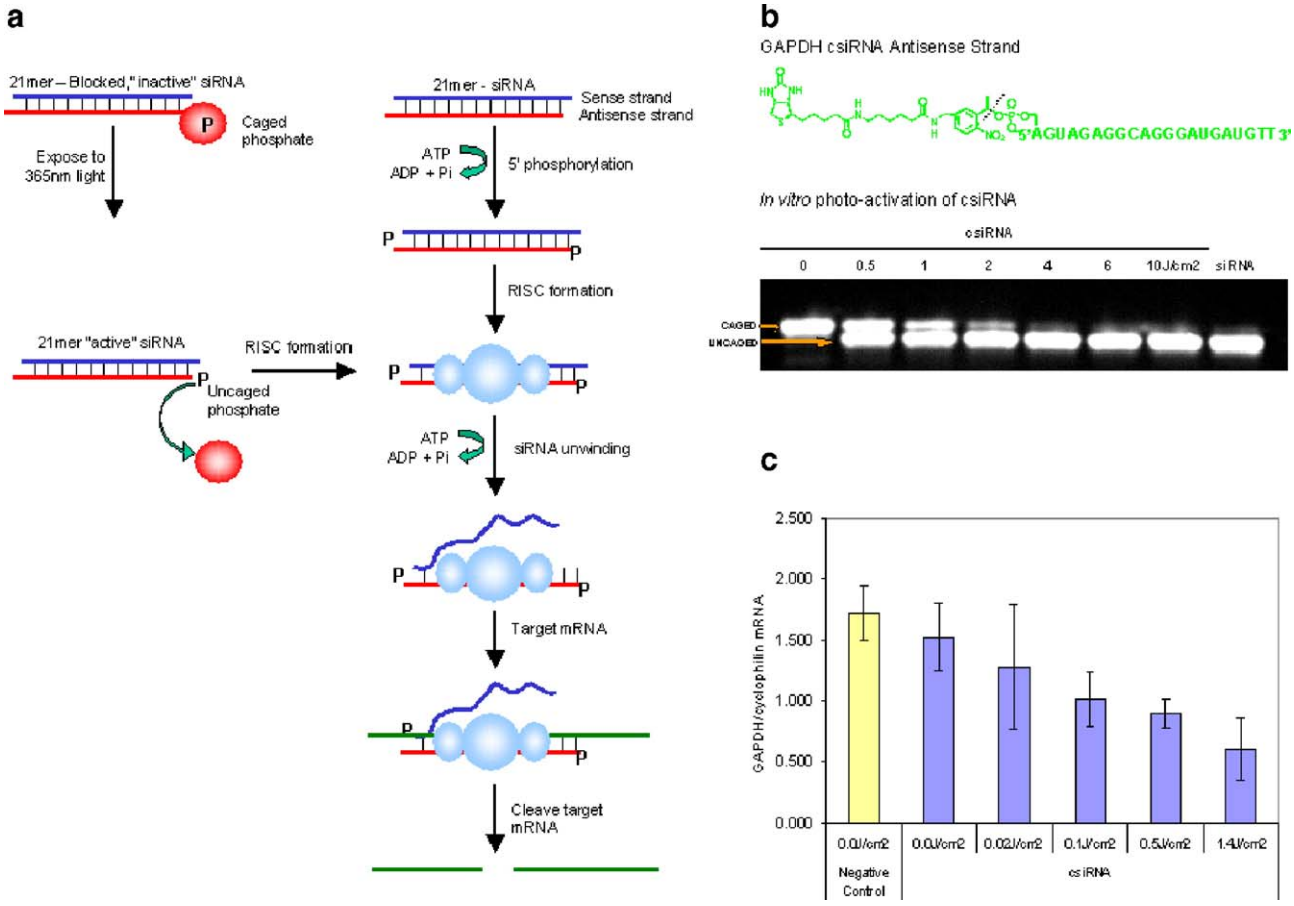

Fig. 1. (a) Schematic showing the principle of light activation of a csiRNA. csiRNA is transfected into cells as an "inactive caged" molecule. Light activation of the csiRNA is made possible by the incorporation of a single NPE (nitrophenyl ethyl) photo removable (cleavable) or "caging" group blocking the access of RISC to the phosphorylated 5′ end antisense strand of the 21-mer siRNA. Once the caging group is removed by a low dose of light (365 nm), the "uncaged active" siRNA assembles with the RISC complex which binds to the complementary mRNA via base pairing interactions to the siRNA antisense strand 5′ end resulting in a sequence-specific degradation of the mRNA and gene silencing. (b) Structure of a GAPDH csiRNA antisense strand. A photo removable (cleavable) "caging" group -biotinylated NPE (nitrophenyl ethyl) was covalently linked to the 5′ phosphate of the 21-mer GAPDH siRNA antisense strands using standard oligoribonucleotide synthesis procedure. The dash line depicts the site of cleavage after light activation. 20 μl of a 5 μM csiRNA solution was pipetted into a clear bottom, black wall plate. The 5 μM solution was exposed to 0–10 J/cm^2 of 365 nm using the UCOM. 6 μl of the solution was loaded into a 1× TBE PAGE gel and electrophoresis at 100 V for 2 h. (c) Dose-dependent gene suppression of GAPDH gene expression by light activated GAPDH csiRNA. HeLa cells were transfected using Lipofectamine for 4 h with 3 nM of the GAPDH csiRNA and scrambled negative control siRNA, and then exposed to 0–1.4 J/cm^2 using the UCOM Microplate Photo-Activator, followed by analysis of mRNA of GAPDH and cyclophilin (QuantiGene) at 24 h post-transfection. GAPDH expression level was normalized to cyclophilin level to correct for differences in cell numbers from well to well. Experiments were performed in triplicates.

and GAPDH mRNA expression levels were measured. Increased knockdown in GAPDH expression levels prior to light activation is viewed as a less efficient csiRNA. According to Table 1, there is increased knockdown of expression as the caging efficiency decreases. This is most likely due to (n-1) residues that make up the majority of impurities from oligonucleotide syntheses. For csiRNA, (n-1) residues are fully active, complete siRNA molecules. The purity of all of csiRNAs are >95% as measured by HPLC, which is achieved on a routine basis and results in maximum of ~30–40% knockdown without light and at least 80% knockdown after exposure to light.

Next, we compared the GAPDH csiRNA light activated before and after transfecting into HeLa cells to a GAPDH siRNA of the same sequence and molarity (Fig. 2a). The siRNA and the in vitro uncaged csiRNA were equally potent in knocking down GAPDH mRNA expression at all times measured. If the csiRNA was not exposed to light, 30–40% suppression of mRNA at 4 h and 24 h post-transfection was detected when compared to the negative control. This 30–40% suppression of mRNA is the result of csiRNA n-1 residues not caged (~5%) and thus are fully active siRNA molecules (as described above and Table 1). At 4 h post-transfection, cells from the same transfection were exposed to light [in vivo csiRNA light activated (T=0)] and cells were immediately lysed. As can be observed the T=0 in vivo uncaged was comparable to the caged csiRNA control without light, since the in vivo uncaged csiRNA was only active for seconds. However, by 24 h and 48 h post-transfection (T=20 h and T=44 h post light activation of cells in vivo, respectively), there was significant mRNA suppression, comparable to the siRNA and the in vitro light activated csiRNA. These results were confirmed at the protein expression level by immunofluorescent analysis (Fig. 2b) and suggested that caged GAPDH csiRNA remained quiescent in the cells until exposed to light.

Table 1
Purity of csiRNA as function of residual activity

Purity of antisense strand csiRNA	csiRNA residual activity (% gene knock down with no exposure to light)[a]
99%	5–10%
97%	10–20%
95%	30–40%
80%	60–70%

Highly purified csiRNA antisense strands are important for successful controllable knockdown experiments. The table shows the correlation between the purity of csiRNA and the activity of csiRNA in cells, measured by HPLC chromatograms of the antisense strand. Different lots GAPDH csiRNA of various purities were transfected into HeLa cells and incubated for 24 h at 37 °C. The cells were not exposed to light in order to keep the csiRNA caged and unreactive. The cells were lysed and GAPDH mRNA expression levels were measured. Increased knockdown in GAPDH expression levels prior to light activation is viewed as a less efficient csiRNA. There is increased knockdown of expression as the caging efficiency decreases. This is most likely due to (n-1) residues that make up the majority of impurities from oligonucleotide syntheses. For csiRNA, (n-1) residues are fully active, complete siRNA molecules. These data are consistent with other csiRNAs used in this study.

[a] % gene knockdown=(1−ratio of normalized mRNA levels of positive/negative csiRNA)×100. All mRNA levels are normalized to cyclophilin level to correct for the differences in cell number from well to well.

We then tested light activated GAPDH csiRNA cotransfected into HeLa cells in the presence of a β-actin siRNA and measured the expression of each mRNA (Fig. 2c). Only cells exposed to light showed suppression of GAPDH mRNA and were equal to cells transfected with either a standard GAPDH siRNA or csiRNA exposed to light. Overall, the β-actin siRNA did not interfere with the light activation of the GAPDH csiRNA and its gene silencing. Using β-actin siRNA in combination with light controllable GAPDH csiRNA, we demonstrated that gene silencing was sequentially activated.

We also tested whether the csiRNA could stimulate nonspecific effects such as activation of the double-stranded RNA (dsRNA) regulated protein kinase PKR and induction of the IFN response. Although the csiRNAs were able to bind to recombinant PKR in vitro whether activated or not (Fig. 3a), they did not induce any detectable activation of PKR as measured by an in vitro kinase assay (Fig. 3b). In accord with this, HT1080 cells transfected with the csiRNA exhibited no evidence of PKR activation as measured by phosphorylation of its downstream target eIF2α (data not shown). Moreover, there was no induction of dsRNA and IFN stimulated protein 56 by the csiRNAs whether caged or uncaged, indicating that they do not activate the IFN system (Fig. 3c). In contrast, a T7 synthesized RNA was able to induce the interferon system as previously reported (Fig. 3c, [13]). The csiRNAs used specifically knocked down the target genes p53 or GAPDH only when exposed to light (Fig. 3c) although the HT1080 cells exposed to light also showed a small but reproducible increase in p53 levels (Fig. 3c and data not shown). Thus, we conclude that csiRNA can specifically induce knockdown of the target genes without activating the IFN system.

We also evaluated the transfection of csiRNA using the novel MPG peptide-based delivery system. We have previously demonstrated that MPG efficiently delivers siRNAs into many different cell types including difficult-to-transfect cells such as differentiated 3T3-L1 cells ([14,15] and data not shown). Fig. 4 shows the photo-activation of GAPDH csiRNA transfected HeLa cells using MPG, where the knock down of GAPDH between normal siRNA and csiRNA are very similar, suggesting that the 5′ photocaging group on csiRNA did not affect the MPG peptide transfection efficiency. We found similar results using lipofectamine transfection reagent (Fig. 2a).

Next, we tested survivin and hNuf2 csiRNAs to induce phenotypes in HeLa cells [16,17]. When compared to the negative scrambled control with light and survivin csiRNA control without light, the light activated survivin csiRNA significantly reduced its expression and increased the number of multinucleated cells, which agrees with previously reported data for HeLa cells under similar conditions (Fig. 5, [16]). Depletion of hNuf2 mRNA in HeLa cells results in mitotic arrest and eventually leads to cell death. Mitotic arrested cells

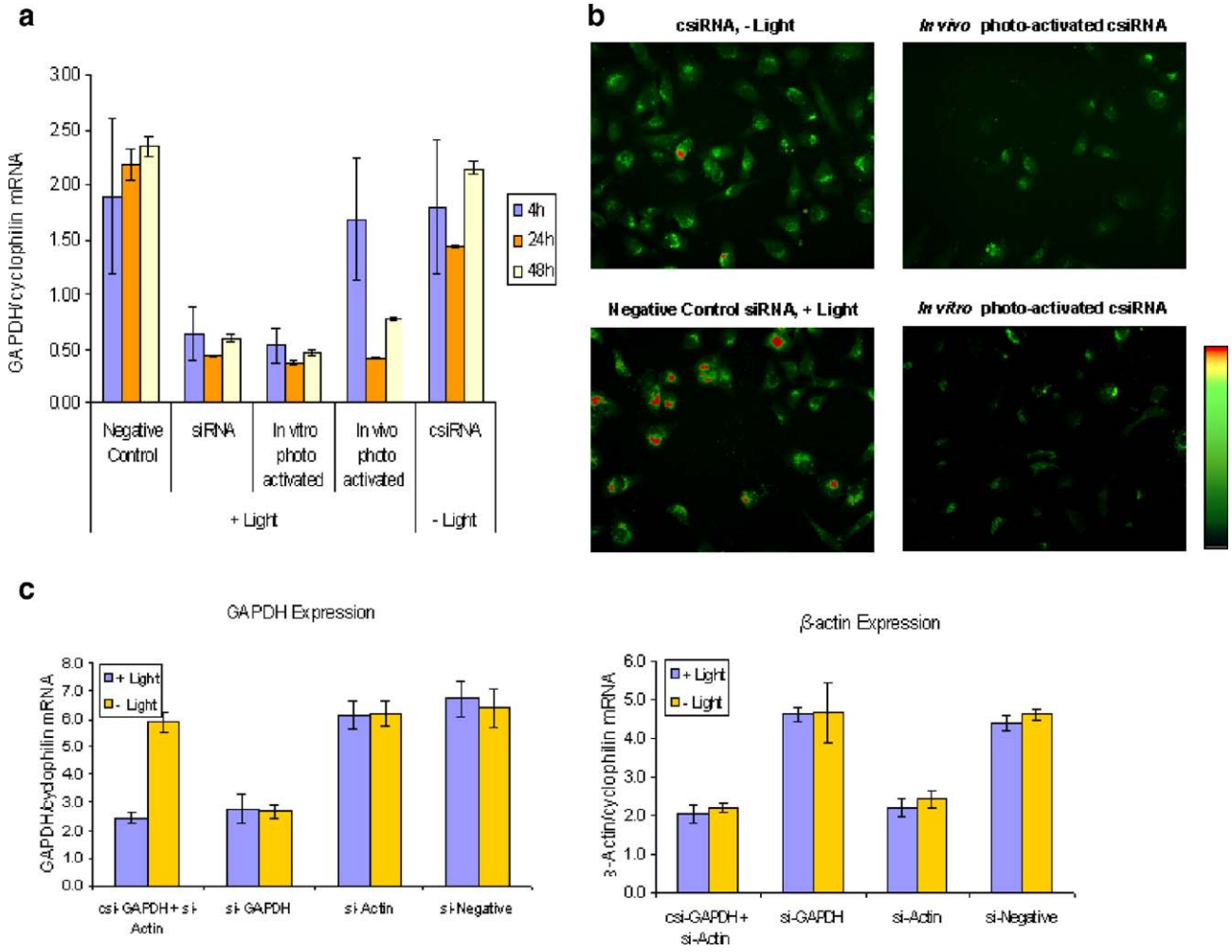

Fig. 2. (a) Photo-activation of GAPDH csiRNA in HeLa cells. HeLa cells transfected with 3 nM of GAPDH siRNA, GAPDH csiRNA or negative control siRNA for 4 h exposed to 1.4 J/cm^2 of light (365 nm) and analyzed for gene expression at 4 h, 24 h and 48 h post-transfection. In vitro=photo-activation of siRNA in solution prior to the start of transfection procedure, In vivo=photo-activation of siRNA in cell after 4 h transfection procedure. (b) Immunofluorescent detection of GAPDH protein. siRNA transfected cells were fixed and probed for expression of GAPDH protein expression using mouse anti-human GAPDH antibody, 48 h post-transfection. (c) Sequential silencing of two genes using a GAPDH csiRNA and a β-actin siRNA. csiRNA GAPDH (2.5 nM) (csi-GAPDH) and siRNA β-actin (62.5 nM) (si-actin), and the negative control (65 nM) siRNA (si-negative) were either co-transfected or individually transfected for 4 h as described in the Materials and methods section. After 4 h transfection, wells were exposed to 2 J/cm^2 (+Light) or 0 J/cm^2 (−Light) followed by mRNA analysis (QuantiGene) at 24 h post-transfection. All mRNA expression levels were normalized to cyclophilin mRNA level to correct for differences in cell number from well to well.

exhibited a round cell phenotype and apoptotic cells showed shrinkage and collapse of both the cell and nuclear membranes [17]. We evaluated hNuf2 csiRNA's capability to photo initiate cell cycle arrest and cell death in HeLa cells and found only light activated hNuf2 csiRNA suppressed its target mRNA expression and exhibited significant numbers of cells of mitotic arrest and apoptotic phenotypes (Fig. 6). In addition, the csiRNA light exposed cells showed considerable reduction of ATP level, suggesting that the cells' energy metabolism was suppressed. On the other hand, higher ATP levels and healthy cells were observed using the negative control siRNA with light or csiRNA without exposure to light. These results agree with those found by Deluca et al. using a standard siRNA [17]. However, we did detect some residual gene suppression (30–40%) in hNuf2 csiRNA transfected cells not exposed to light (Fig. 6), which may be due to csiRNA molecules not blocked during oligosynthesis and not completely removed by our purification procedure (<5%) as described above. The hNuf2 mRNA is expressed at only 10–20 copies/cell and thus picomolar concentrations (5% of 3 nM transfected) may be sufficient to cause the 30–40% knockdown. Nonetheless, we did not detect the phenotype. As can be observed in Fig. 7, using the hNuf2 csiRNA and increasing the light energy, we demonstrated a dose-dependent induction of the expected phenotypes, where 0.3 J/cm^2 was enough energy to fully induce cell cycle arrest and cause apoptosis.

Temporal and/or spatial regulation of gene expression is important for many phenotypic assays [10]. Recently, inducible promoter systems driving expression of shRNAs in mammals and plants have been reported [10,18]. However, these systems require more manipulation, making them more applicable for in vivo experimentation than for in vitro testing in cultured cell assays. In addition, stable cell lines can have basal expression in absence of the inducer, making them

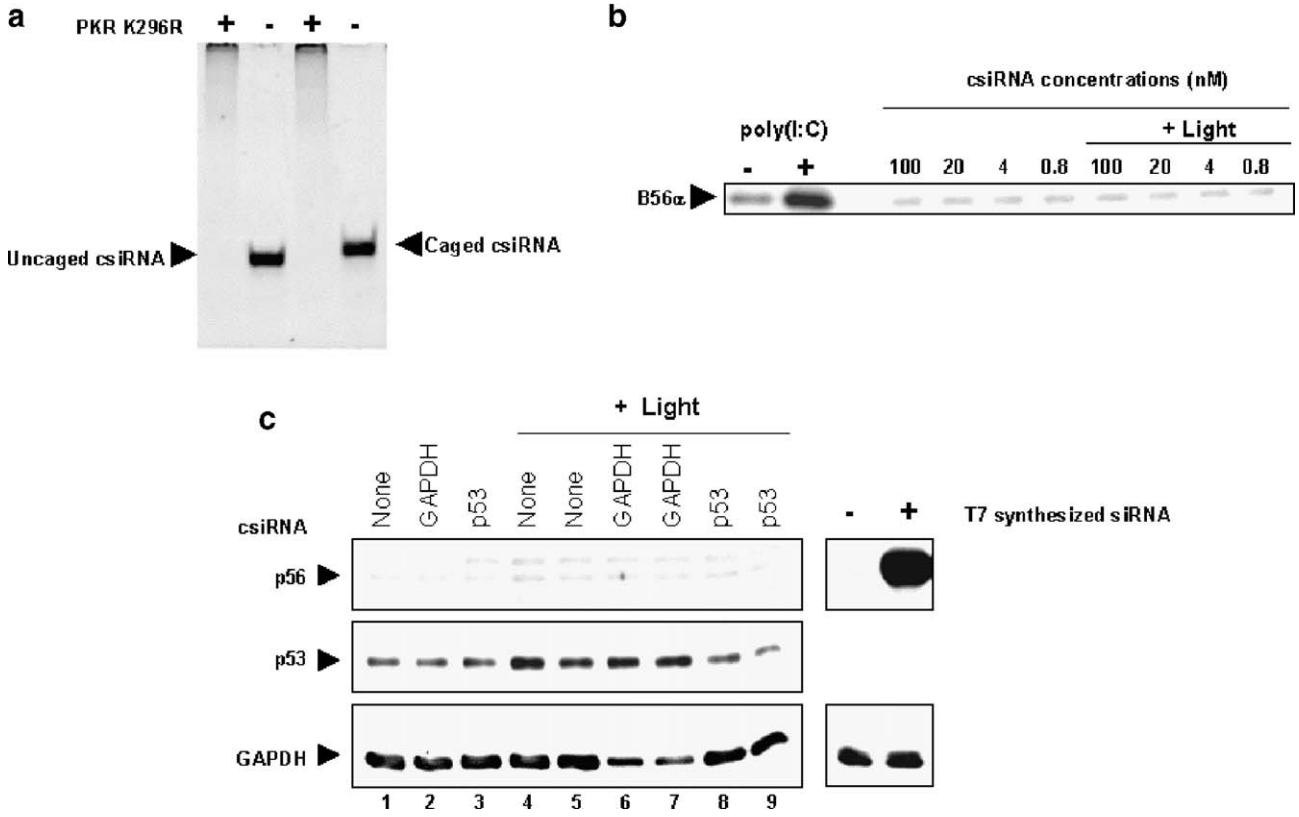

Fig. 3. (a) The ability of the siRNAs, caged or uncaged, to bind PKR was analyzed by electrophoresis mobility shift assays (EMSA). The siRNAs were incubated with recombinant PKR (K296R), separated on a native PAGE and stained with SYBR Gold to visualize the nucleic acids. (b) Caged or uncaged siRNAs were tested for their capacity to activate PKR in vitro. The kinase assay was performed with recombinant PKR and activity was determined by phosphorylation of B56 alpha as a substrate (30). poly(I:C) was used as a positive control for activation of PKR. (c) Caged or uncaged csiRNAs, were tested to determine whether they would activate the IFN system in cell culture. The csiRNAs, targeting GAPDH or p53, were transfected into HT1080 cells at a final concentration of 30 nM using Lipofectamine 2000. After 4 h, the transfection mix was discarded and new media added. The cells were exposed to 1.4 J/cm^2 of light (365 nm) where indicated (+Light). After 40 h, whole cell lysates were prepared and analyzed by Western blot with the indicated antibodies.

"leaky" and some inducers may cause toxic or off target effects on cells, making them not desirable for many phenotypic assays [10].

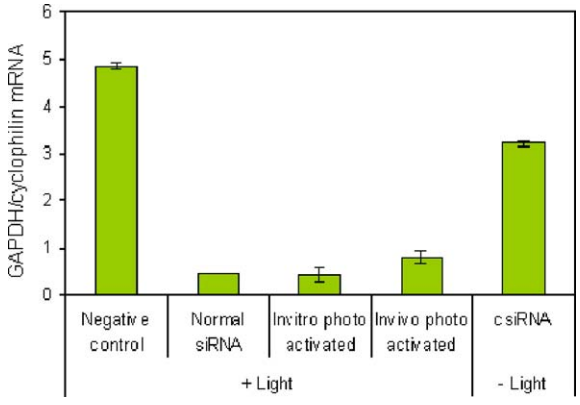

Fig. 4. MPG delivery of GAPDH csiRNA into HeLa cells. HeLa cells transfected with 3 nM of GAPDH siRNA, GAPDH csiRNA or negative control siRNA for 4 h exposed to 1.4 J/cm^2 of light (365 nm) and analyzed for gene expression at 24 h post-transfection. In vitro=photo-activation of siRNA in solution prior to the start of transfection procedure, In vivo=photo-activation of siRNA in cell after 4 h transfection procedure. Note: The in vivo photo-activation represents 20 h knockdown, since the activity was released 4 h post-transfection.

A variety of light activated or "caged" molecules have been used extensively in cell biology and pharmacology including secondary messengers, RNA, DNA, peptides and proteins [12,19–25]. Recently, a siRNA was caged post-synthesis using 4, 5 dimethyoxy-2-nitrophenylethyl (DMNPE) to create a diazo compound that reacts randomly with multiple internal phosphate groups of the siRNA [25]. Depending on the caging group equivalents per siRNA molecule, either the siRNA's knock-down activity was partially blocked (~50%) or only partially activated (~50%) by light, even when increasing the exposure times, where longer irradiation resulted in phototoxicity. In this study, we have demonstrated an alternative approach to control the activity of a siRNA resulting in regulated gene silencing with phenotypes. We found incorporating a single NPE (nitrophenyl ethyl) caging group at the 5′ end of a siRNA antisense strand was sufficient to block enough of its activity in cells and was totally removed using a 2 J/cm^2 of light (365 nm) energy to induce phenotypes. At this energy level, we have not detected any effects on cell viability (ATP levels, caspase 3 activity, membrane integrity, morphology), including light sensitive cells (e.g., Jurkat cells), which is in agreement with previous results [12]; however, we did observe small increases of p53 in HT1080 cells. We observed a minimum of 12 J/cm^2 to induce apoptosis, which is in accordance with the findings that

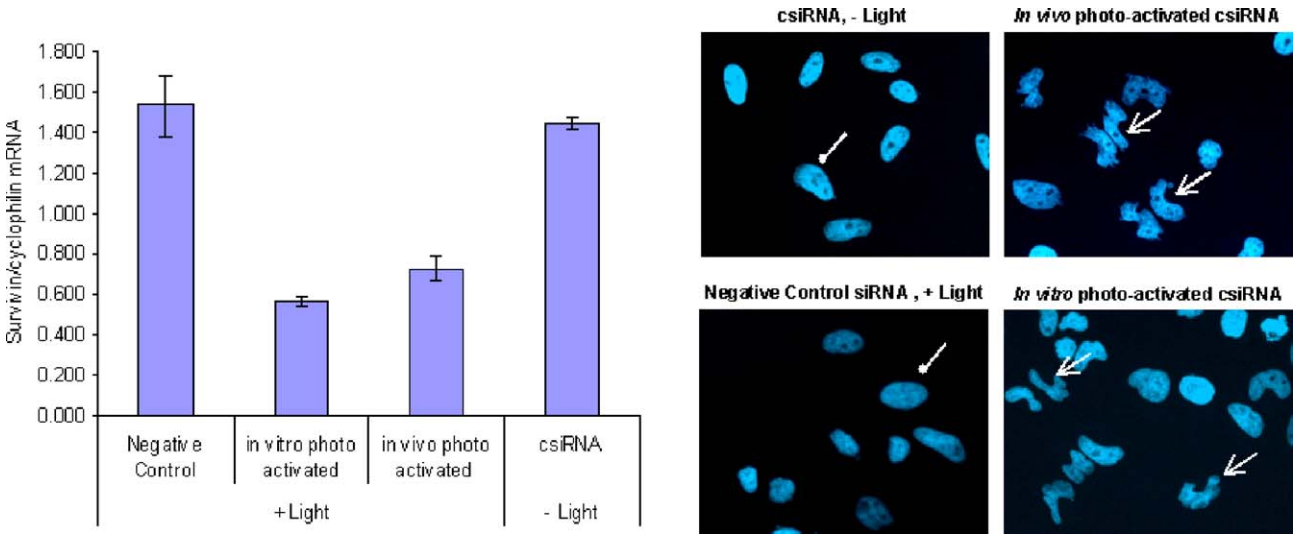

Fig. 5. Light activation of survivin csiRNA in HeLa cells. (Left) HeLa cells were transfected with 3 nM of survivin csiRNA, exposed to 2 J/cm^2 of light 4 h post-transfection, lysed 24 h post-transfection and mRNA level quantitated. In vitro=photo-activation of siRNA in solution prior to transfection, In vivo=photo-activation of siRNA in cells after 4 h post-transfection. (Right) Nuclear stain of HeLa cells was performed using DAPI at 24 h post-transfection. Multinucleated cells (arrow) are observed in wells transfected with survivin csiRNA and exposed to light, whereas round nuclei (line) are observed in wells transfected with csiRNA and not exposed to light or with the negative control siRNA exposed to light.

27 J/cm^2 of light (365 nm) induced apoptosis in keratinocytes [26] and intracellular free Ca^{2+} was released in Jurkat cells exposed to 10 J/cm^2 of light [27]. Using commercially available transfection reagents, we found that csiRNAs are comparable to a standard siRNAs in their transfection efficiency and potency of gene silencing. To date, we have successfully photo-activated

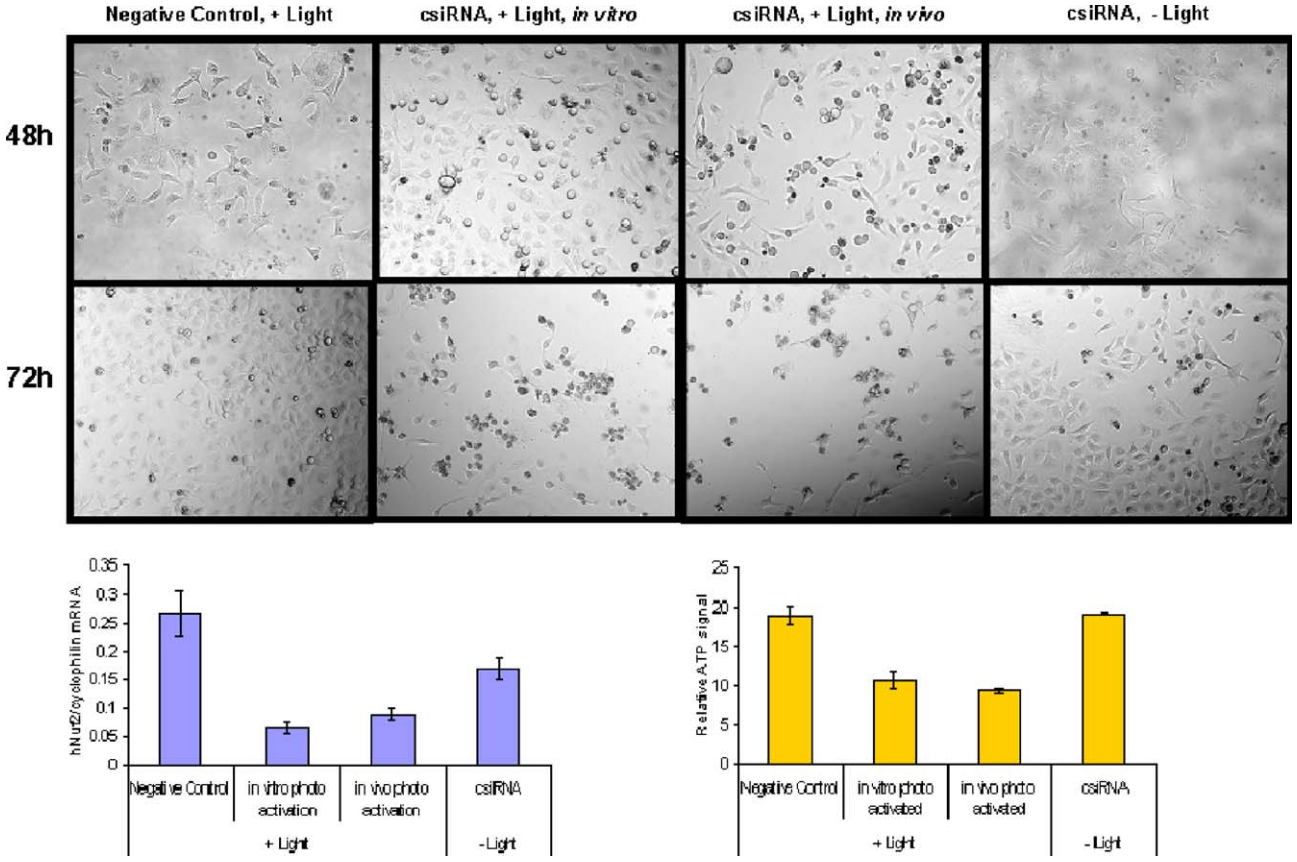

Fig. 6. Activation of hNuf2 csiRNA and mitotic arrest in HeLa cells. HeLa cells were transfected with 3 nM of hNuf2 csiRNA, exposed to 2 J/cm^2 of light at 4 h post-transfection and bright field images were taken at 48 h and 72 h post-transfection (top). mRNA (left) level and ATP/Cell Viability Assay (right) were performed at 48 h or 72 h post-transfection, respectively.

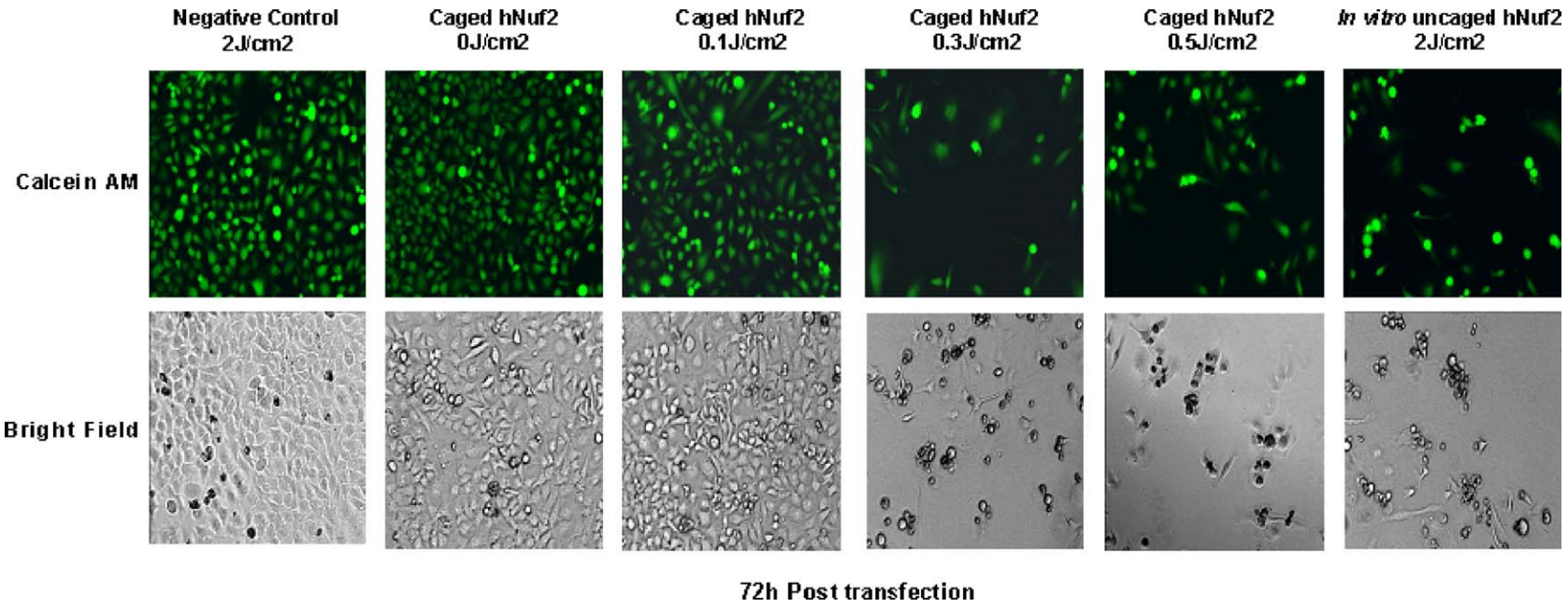

Fig. 7. Dose response of hNuf2 csiRNA using light. HeLa cells were transfected with 3 nM of hNuf2 csiRNA, exposed to 0, 0.1, 0.3, 0.5 and 2 J/cm^2 of light at 4 h post-transfection. Bright field images (bottom) and intracellular Calcein AM esterase activity of cells (top) were obtained at 72 h post-transfection.

csiRNAs 24 h post-transfection and achieved knockdown of target mRNAs (data not shown). The half-life of a csiRNA is equivalent to that of a siRNA and siRNA half-life depends on the number of cell divisions (dilution effect) and overall ribonuclease activity of transfected cells. To achieve longer-term stability against ribonucleases, internal ribonucleotides of the csiRNAs can be modified as previously demonstrated for siRNAs [28,29].

The data presented here demonstrated that light could precisely control csiRNA gene silencing and the resulting phenotypes in living cells. The technology enabled a $T=0$ time point and the csiRNA served as its own control. In addition, by controlling the light energy, csiRNA was activated such that a dose-dependent response of mRNA suppression was achieved. Controllable siRNA (csiRNA) opens the door to studying toxic and off-target events independently of cellular transfection, enabling kinetic measurements in cells and the induction or modulation of phenotypes. Finally, csiRNA could be used in combination with GFP fused proteins to demonstrate protein suppression and associated phenotype in living cells.

3. Materials and methods

3.1. csiRNA and siRNA synthesis

The following human csiRNAs and siRNAs were synthesized using commercially available phosphoramidite monomers:

GAPDH: 5′-CAUCAUCCCUGCCUCUACUTT-3′ (sense strand)
survivin: 5′-GGAACAUAAAAAGCAUUCGTT-3′ (sense strand)
hNuf2: 5′-AAGCATGCCGTGAAACGTATT-3′ (sense strand)
p53: 5′-GGAAAUUUGCGUGUGGAGUTT-3′ (sense strand)

β-actin and GAPDH negative control siRNAs were obtained from Ambion. Photo removable (cleavable) phosphoraimidite, [1-*N*-(4, 4′-Dimethoxytrityl)-5-(6-biotinamidocaproamidomethyl)-1-(2-nitrophenyl)-ethyl]-2-cyanoethyl-(*N*,*N*-diisopropyl)-phosphoramidite was obtained from Glenn Research. It was incorporated into 5′ phosphate group of the antisense strand of a 21-mer siRNA using the standard oligoribonucleotide synthesis procedure. The modified 21-mer antisense strand was purified using RNAse-free HPLC and verified by gel electrophoresis and mass spectrometry. The purity was routinely >95%. The modified antisense strand was annealed to the sense strand by heating equal molar of both strands in the annealing solution (250 mM Tris, pH 7.4, 500 mM NaCl, 5 mM EDTA) at 90 °C for 5 min. The solution is allowed to cool for 4 h at room temperature.

3.2. Transfection of csiRNAs

HeLa and HT1080 cells were transfected using Lipofectamine 2000 (Invitrogen), Oligofectamine (Invitrogen) and/or MPG "Express-si" (Genospectra) according to the manuals. Briefly, approximately 2000–5000 cells/well were grown overnight in a clear bottom, black well 96-well microplate (Costar). Cells were incubated with csiRNA or siRNA for 4 h and then replaced with complete growth media. HT1080 cells were transfected using the same protocol in 12-well plates and whole cell lysates were prepared for Western blot analysis.

3.3. Sequential silencing of GAPDH csiRNA and β-actin siRNA

The csiRNA GAPDH (csi-GAPDH, Genospectra), siRNA β-actin (si-actin, Ambion) and negative control GAPDH siRNA (si-negative, Ambion) were thawed on ice and diluted to 5 μM stock concentrations in 1× dilution buffer (5 × siDilution Buffer, Genospectra). For each csiRNA or siRNA, 15 μl of stock siRNA-Actin, 0.6 μl stock csiRNA-GAPDH or 15.6 μl of siRNA-negative (Ambion) was diluted in 106 μl (final volume) of Opti-MEM (Invitrogen) at room temperature and incubated for 5–10 min. For cotransfection, final concentration per well was 62.5 nM siRNA β-actin and 2.5 nM csiRNA-GAPDH. For single siRNA transfection, final concentration was 62.5 nM si-actin, 2.5 nM csiRNA-GAPDH or siRNA-GAPDH, and 65 nM siRNA-negative. Oligofectamine (Invitrogen) was warmed to room temperature and mixed according to the manufacturer's instructions. For each well assayed 6 μl were diluted in 30 μl Opti-MEM [final volume, (Invitrogen)] and incubated at room temperature for 15–20 min. To form transfection complexes, 30 μl of diluted Oligofectamine (Invitrogen) were added to each tube of diluted siRNA and incubated for 30 min at room temperature. After the 30-min incubation period, 64 μl of additional Opti-MEM (Invitrogen) were added to each tube and the tube gently mixed by inverting. 20 μl of diluted complexes were added directly to wells containing cells in complete growth medium ($T=0$). The final volume in each well is 120 μl. At $T=4$ h post-transfection, complexes were removed and replaced with 120 μl of fresh complete growth medium. The plates were exposed to 2 J/cm^2 of 365 nm light using the UCOM Microplate Photoactivator (Genospectra) and then returned to the incubator. Replicates of 3 wells were run for all conditions tested. At $T=24$ h post-transfection, cells were lysed with 60 μl of QuantiGene lysis buffer and mRNA levels for GAPDH, β-actin and cyclophilin (internal control gene) were measured using the QuantiGene Reagent System (Genospectra). All mRNA expression levels were normalized to cyclophilin mRNA levels to correct for differences in cell number from well to well.

3.4. In vivo photo activation of csiRNA

After transfection, cells were exposed to 0.02–2.0 J/cm^2 (5–50 s) of 365 nm ± 20 nm light using a UCOM Microplate Photo-Activator (Genospectra) according to the UCOM and csiRNA manuals. The UCOM is designed specifically for photo-activation and photo-affinity applications. The UCOM generates light that is uniformly distributed across a standard microplate. The large area of illumination allows simultaneous and high throughput release of photo-activated samples in a microplate format. To test potency of a new csiRNA (>95% purity), we routinely transfect cells with increasing amounts of csiRNA (1–30 nM) without light induction until 10–30% mRNA knockdown is observed. Once the concentration of csiRNA is established to initiate knockdown of mRNA without light, it is our experience that at least 80% knockdown is observed with light induction.

3.5. Cell viability assay

Cell viability was determined by measuring the ATP levels using the Cell Titer-Glo Assay (Promega) according to the manual.

3.6. Quantitation of mRNA

The cells were lysed and mRNA expression directly measured using the Branch DNA Technology "QuantiGene Reagent System" (Genospectra) according to the manual. No mRNA purification and amplification are required for this assay. Duplicate or triplicate wells were run to obtain average and standard deviation values. The difference in cell number per well was corrected by calculating the ratio of siRNA-targeted gene to an internal control gene cyclophilin.

3.7. Cells and reagents

HT1080 cells were grown in 10% FBS DMEM. Antibodies against human p53 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and GAPDH from Chemicon International (Temecula, CA). Antibodies against p56 were a gift from Ganes Sen (Cleveland Clinic Foundation).

3.8. Immunofluorescent detection of GAPDH

Cells were washed with PBS and fixed with 4% formaldehyde for 4 min. Cells were washed with PBS, treated with 0.1% triton X-100 for 5 min and blocked with 5% dry milk for 30 min and incubated in 1:2000 dilution of mouse

anti-human GAPDH (Abcam) for 1 h. After a PBS wash, fixed cells were incubated in 1:1000 dilution of goat anti-mouse Alexa-488 conjugate (Molecular Probes) for 45 min. Finally, after a PBS wash, cells were viewed under a fluorescent microscope.

3.9. Western blot analyses

Briefly, cells were lysed in 50 mM Tris buffer, pH 7.4 containing 150 mM of NaCl, 50 mM of NaF, 10 mM of β-glycerophosphate, 1% Triton X-100, 0.1 mM of EDTA, 10% glycerol and protease/phosphatase inhibitors. The samples were kept on ice for 10 min, vortexed and centrifuged for 15 min at 14000 rpm, the supernatant collected in a new tube and protein concentrations determined using the Protein assay kit (Bio-Rad). 30 g of total protein were separated on SDS-polyacrylamide gels (PAGE), transferred to Immobilon™-PSQ membranes (Millipore) and probed with the indicated antibodies.

3.10. Kinase activity assay

100 ng of purified PKR was incubated in kinase buffer (10 mM Tris–HCl [pH 7.6], 50 mM KCl, 2 mM Mg acetate, 7 mM 2-mercaptoethanol, 20% glycerol, 1 mM $MnCl_2$) in the presence of 10 mM ATP and 0.1 μCi/μl [γ-^{32}p]-ATP. The siRNAs were added to the kinase assay at different concentrations and the kinase activity of PKR was inspected by including 100 ng of recombinant B56α prepared as described previously [30]. 1 ng/μl of poly(I:C) was used as a positive control. The reaction was incubated for 30 min at 30 °C, the proteins resolved by SDS-PAGE and the gel autoradiographed.

3.11. Survivin phenotypic assay

HeLa cells were transfected with the human survivin csiRNA or in vitro photo-activated csiRNA using Lipofectamine 2000 as described above. The HeLa cells transfected with the csiRNA were photoactivated with light 4 h post-transfection, fixed using 4% paraformaldehyde at 24 h post transfection and the nuclei were stained using DAPI dye (Calbiochem). Quantitation of multinucleated cells was performed by counting the total number of fragmented multi-nuclei (arrow) and round nuclei (line) from two fields per fluorescent image.

3.12. hNuf2 phenotypic assay

HeLa cells were transfected with human hNuf2 csiRNA or in vitro photo activated csiRNA for 4 h using Lipofectamine 2000 as described above. At 48 h and 72 h post-transfection, bright field images of transfected cells were taken. To measure cell viability, Calcein AM (Molecular Probes) was used to detect esterase activity in cells. Images were taken at 48 h post-transfection.

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