Antibody-coupled siRNA as an efficient method for *in vivo* mRNA knockdown

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Knockdown of genes by RNA interference (RNAi) *in vitro* requires methods of transfection or transduction, both of which have limited impact *in vivo*. As a virus-free approach, we chemically coupled cell surface receptors internalizing antibodies to the short interfering RNA (siRNA) carrier peptide protamine using the bispecific cross-linker sulfo-SMCC (sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate). First, protamine was conjugated amino-terminally to sulfo-SMCC, and then this conjugate was coupled via cysteine residues to the IgG backbone to carry siRNA. This complex can efficiently find, bind and internalize into receptor-positive cells *in vitro* and *in vivo*, which can be checked by flow cytometry, fluorescence microscopy and western blotting. This method obtains results similar to those of siRNA targeting molecules engineered by genetic fusions between receptor-binding and siRNA carrier units, with the advantage of using readily available purified proteins without the need for engineering, expression and purification of respective constructs. The procedure for coupling the complex takes ~2 d, and the functional assays take ~2 weeks.

INTRODUCTION

Development of the protocol

We saw the necessity to develop a method that protects siRNA from digestion and that specifically internalizes this siRNA into (tumor) cells[1]. Developing a strategy to apply siRNAs *in vivo* is a challenge, because the molecules, which are highly charged and short-lived, often fail to be taken up by tumor cells after systemic application. To enhance protection against enzymatic digestion, siRNAs have been coupled to substances such as cationic lipids, nanoparticles, exosomes or peptides[2–5]. Although these methods provide protection for the siRNA, they do not guide the siRNAs specifically toward tumor cells unless they are equipped with cell-specific targeting signals such as the integration of rabies virus glycoprotein (RVG)-derived peptides into exosomes for brain-specific delivery of exosome-bound siRNA[3] or the surface binding of coated cationic liposomes with anti-GD2 antibodies for neuroblastoma-specific delivery of siRNA[6]. Similarly to previous reports, we also chose the most obvious method for achieving the molecular guidance of systemically applied siRNA: an antibody raised against a characteristic cell surface receptor[5,7]. In contrast, however, our siRNA delivery protocol does not rely on the encapsulation of siRNA in complex vesicular structures; rather, it relies on the complexation of siRNA by a natural nucleic acid–binding peptide, protamine[2,8], which is conjugated to the targeting antibody. We propose to use this method in a scientific setting to deliver siRNA to specified cells via antibody binding, internalizing the recognized receptors and knocking down target gene products, and later in the clinic to silence oncogenes driving malignant disease as an individualized cancer treatment in a tumor cell–specific manner. We decided to develop a new receptor-specific approach to silencing mRNAs encoding user-defined genes by RNAi. To transport siRNA by, e.g., the monoclonal anti-epidermal growth factor receptor (anti-EGFR) antibody cetuximab[9], we conjugated the antibody to the cationic peptide protamine using the cross-linker sulfo-SMCC[1]. Protamine is known to bind nucleic acids by electrostatic interactions[2,10], and it was shown to bind siRNA molecules, using other methodologies involving conjugation[8] or genetically engineered fusion proteins[11,12]. Our anti-EGFR-antibody-protamine conjugate (**Figs. 1–3a**) was capable of binding to, and internalizing with, the EGFR, which can be followed by flow cytometry (**Fig. 3b**) and fluorescence microscopy (**Fig. 4**, panel c). We treated different cell lines with the cetuximab-protamine carrier loaded with Kirsten rat sarcoma viral oncogene homolog (KRAS)-specific endoribonuclease-prepared siRNA (esiRNA), and we subsequently observed efficient knockdown of KRAS, as determined by western blot analysis[1] (**Fig. 5**). esiRNAs are enzymatically generated from a long double-stranded RNA *in vitro*. We chose esiRNAs to knock down KRAS expression because the heterogeneous pool of siRNA-like molecules in esiRNAs ensures efficient targeting of the targeted transcript while avoiding off-target effects[13].

The antibody-esiRNA complexes can be injected systemically to mice transplanted with human EGFR-positive tumor cells; they can mark and enter the tumor cells specifically and yield a systemic targeting efficacy[1] (**Fig. 6**).

As another example for the technology, to deliver siRNA to cells that are negative for EGFR, but positive for insulin-like growth factor 1 receptor (IGF1R), we used a mouse monoclonal antibody (mAb) raised against IGF1R, which is a frequently discussed target molecule on solid tumors, especially sarcomas[14,15] (**Fig. 7**). Similarly to the EGFR-specific antibody cetuximab mentioned previously, this antibody binds the respective receptor, blocks ligand-dependent signaling and leads to the internalization of the receptor plus the antibody[16,17].

EGFR-positive cell lines, as used in our example, are numerous and, as we did not observe a linear relation between the expression level of EGFR and the knockdown efficiency after treatment, it is worth testing any EGFR-positive cell type representing various

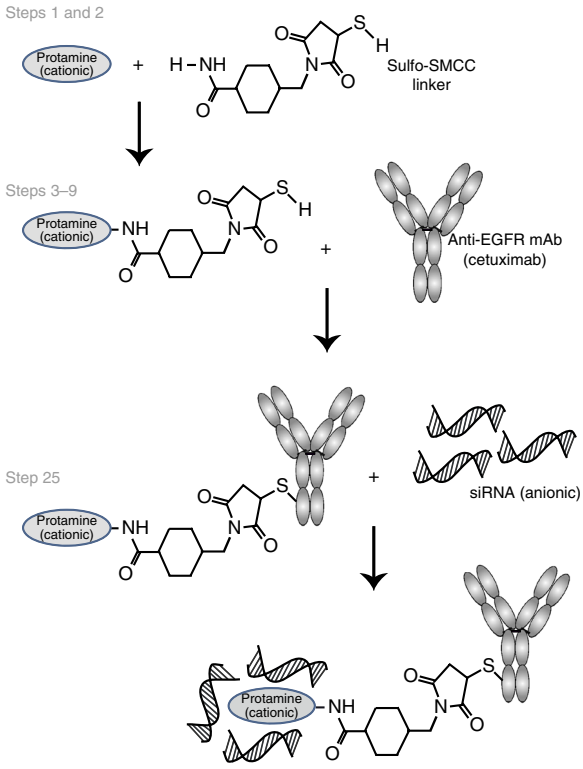

Figure 1 | Composition of the antibody-siRNA complex. First, the siRNA-binding moiety protamine and the conjugation moiety sulfo-SMCC bind covalently via an amino group (Steps 1 and 2). Second, the monoclonal anti-EGFR-antibody cetuximab (anti-EGFR mAb) is coupled to sulfo-SMCC-protamine by a cysteine residue within the antibody (Steps 3–9). Last, the (e)siRNA binds to the protamine within the cetuximab–sulfo-SMCC–protamine (CSP) complex (CSP) by electrostatic interactions (Step 25). Please note that **Box 1** describes an alternative procedure for treating IGF1R-positive cell lines.

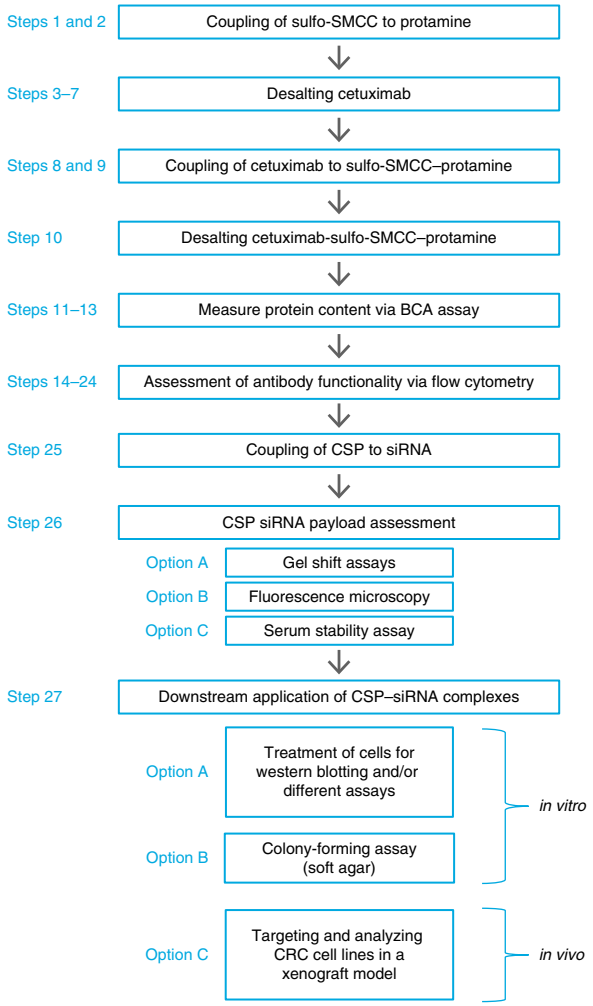

Figure 2 | Flowchart illustrating the protocol to form and use the CSP–siRNA complexes. Please note that **Box 1** describes an alternative procedure to treat IGF1R-positive cell lines.

histologies, regardless of expression level. As an alternative line of targeting, IGF1R may be used as a targetable receptor to systemically deliver siRNA to IGF1R-positive but EGFR-negative cells.

Applications of the method

Anti-EGFR–antibody–siRNA complexes can be applied for all EGFR-positive cell types and coupled to all short duplex siRNA as desired. Many cell types express EGFR on their surfaces, such as HeLa cells, LoVo cells and a multitude of colorectal tissue–, soft tissue– and bone tissue–derived, or lung tumor–derived cell types. Moreover, this method can be applied to other antibodies that include reduced cysteine residues, which can be coupled to SMCC and protamine, as shown here by the example of the IGF1R-specific antibody GR11L (GR11L is the Merck Millipore catalog no.; see **Box 1**). Therefore, the use of antibody-siRNA complexes can be widely applied to many cell types and any siRNA. For *in vivo* application, such as cancer therapy, one has to consider that the more specifically the antibody binds to a cancer cell–specific surface protein, the less specifically the oncogene-interfering siRNA can be chosen. In contrast, siRNA that downregulates a cancer cell–specific oncogene that does not exist in healthy cells can potentially be carried by a rather unspecific binding antibody. However, the therapeutic principle presented here carries the possibility of dual specificity with the opportunity for fine-tuning according to the therapeutic needs and the combination possibilities of entry molecules with oncogenic driver targets.

In addition to the application as a therapeutic agent, these antibody-siRNA complexes might also help downregulate genes in cells that are rather difficult to transfect—i.e., stem cells or primary cells or, more generally speaking, nonadhesive cells.

Comparison with other methods

The advances of lipofection and the use of polycationic substances[5] are impressive, but they do have limited impact for *in vivo* applications, as they do not exhibit a cellular targeting specification. The expression of shRNA through viral systems allows the efficient transduction of cell lines *in vitro*, and in preclinical *in vivo* studies[18], but it also lacks a cell-specific determination component.

The application of therapeutic siRNAs in clinical situations was an ever-increasing field over the past decade. The topical application of naked siRNAs to the eye in age-related macular degeneration (AMD)[19] or administration by inhalation in respiratory virus infections[19] have proven to be successful. In contrast, the systemic application of siRNAs was always hampered by high immunogenicity, renal clearance, and susceptibility to enzymatic digest, and consequently a low targeting efficiency to the cell of

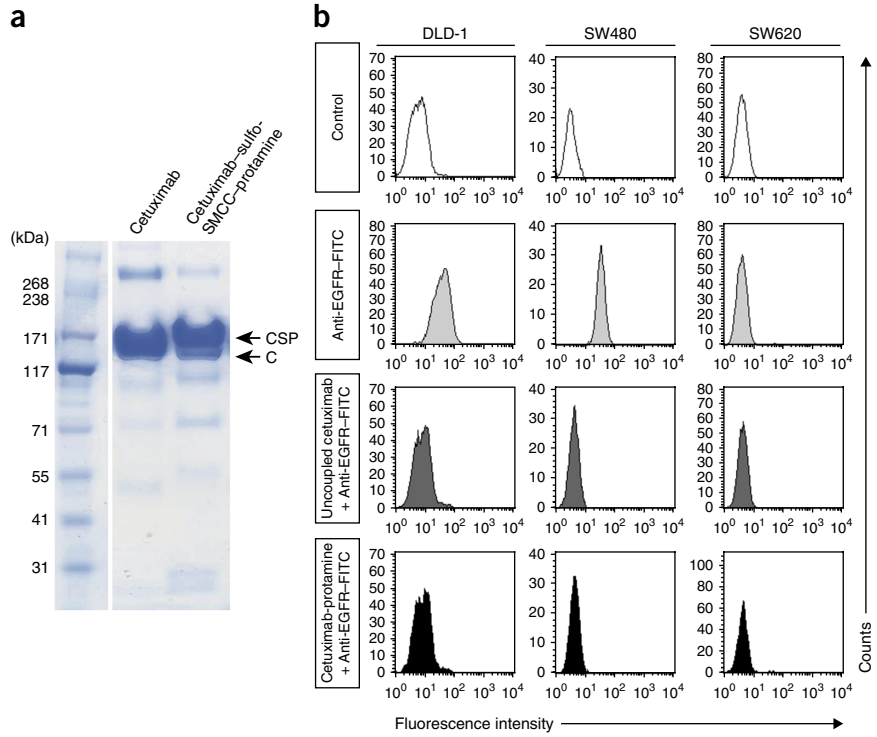

Figure 3 | Quality control assays for functional antibody-siRNA complexes. (**a**) SDS-PAGE showing the efficient binding of the EGFR-specific antibody cetuximab ('C') to protamine via the sulfo-SMCC linker (CSP). Note the molecular weight increase in the CSP lane. (**b**) Before using an antibody as a carrier for siRNA delivery, efficient internalization of the antibody by the respective receptor should be checked (Steps 14–24). This can be determined by flow cytometry (FACS analysis). Here we analyzed the expression of EGFR on the surface of DLD-1 (left column), SW480 (middle column), and EGFR-negative SW620 cells (right column) as a negative control by flow cytometry before and after incubation with uncoupled cetuximab (third row) and with cetuximab coupled to protamine (bottom row). EGFR surface expression was detected using a FITC-labeled EGFR-specific antibody (anti-EGFR–FITC; Santa Cruz Biotechnology, cat. no. sc-120-FITC) that binds to a different extracellular epitope of EGFR than cetuximab. The cell lines DLD-1 and SW480 showed high EGFR expression on their surfaces (anti-EGFR–FITC, second row) in comparison with a control antibody (control, top row). EGFR expression was no longer detectable because of internalization of the receptor after treatment with uncoupled cetuximab (uncoupled cetuximab + anti-EGFR, third row) and CSP (cetuximab-protamine + anti-EGFR–FITC, bottom row), as shown by flow cytometry. As a control, the SW620 cell line (right) showed no EGFR surface expression using anti-EGFR–FITC. This illustrates the efficient internalization of the receptor into EGFR-positive cells upon antibody binding, which indicates that the cells will also take up the antibody in complex with the siRNA. The *y*-axis depicts cell counts, and the *x*-axis depicts fluorescence intensity.

interest. This was partially circumvented by the use of nanoparticles[2] or lipid spheres[20], or even exosomal transport[3], in order to protect the siRNA load in clinical trials. However, a cell-specific determination component has to be introduced here to provide a specific direction for the carrier system, leading it toward the cell of interest[4]. The alternative biotechnological methods to create and produce fusion proteins of antibodies or ligands with siRNA carriers by recombinant DNA technology[11] should be explored further. For instance, genetic fusion to cationic peptides such as poly-arginine or protamine[8] in bacterial

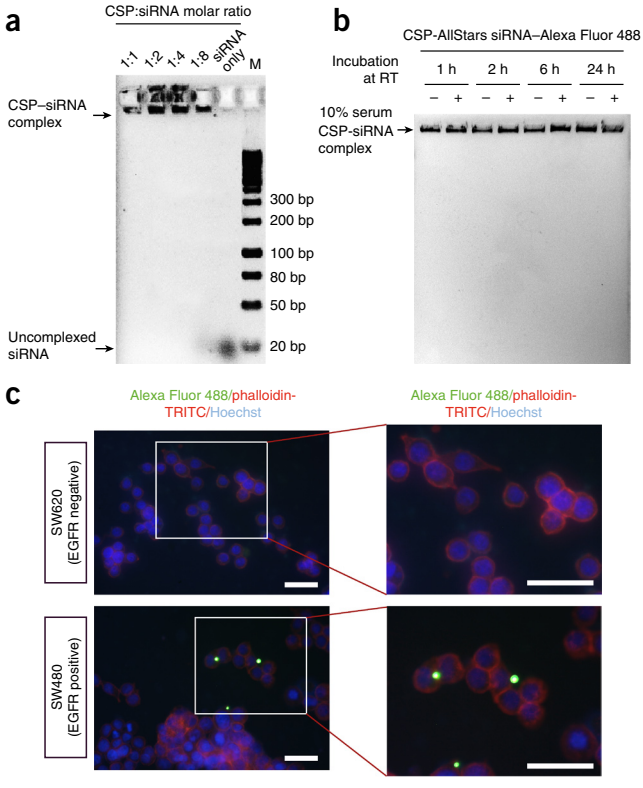

Figure 4 | Quality control, binding and cellular uptake. (**a**) Specific siRNA payload estimation of CSP–siRNA complexes. Different molar ratios of CSP complexes to Alexa Fluor 488–siRNA (here: AllStars negative control siRNA–Alexa Fluor 488, Qiagen) were incubated as described in Step 26A(i,ii) and loaded on a 1.5% (wt/vol) agarose gel, and the band shift was then documented. At a molecular excess of 1:1–1:4, siRNA duplexes are completely retarded by CSP. At a molecular excess of 1:8, CSP is saturated and unbound siRNA duplexes start to appear. The right lane depicts uncomplexed siRNA without CSP addition. M, molecular weight marker. (**b**) Testing the protective effect of CSP for siRNA against enzymatic digest. Samples of siRNA were bound to CSP and exposed to 10% (vol/vol) FCS for time spans ranging from 1 to 24 h. Consequently, the samples were loaded to an agarose gel and subjected to electrophoresis followed by ethidium bromide visualization of the bound siRNA. The conjugated protein–siRNA complex was detectable as a high-molecular-weight band showing only minor intensity changes after serum exposition. (**c**) Internalization of fluorescent Alexa Fluor 488–siRNA using two different cell lines. Top, EGFR-negative SW620 cells were incubated with CSP coupled to fluorescent Alexa Fluor–488 siRNA. Bottom, EGFR-positive SW480 cells were incubated with CSP coupled to fluorescent Alexa Fluor 488–siRNA. After incubation, both cell lines were fixed and stained with phalloidin-TRITC to visualize the cytoskeleton and with Hoechst to visualize the nucleus. Only in the EGFR-positive SW480 cells can internalized Alexa Fluor 488–siRNA vesicles with a perinuclear localization in the cytoplasm (green) be seen. The EGFR-negative SW620 cells do not show these vesicles. Scale bars, 20 μm.

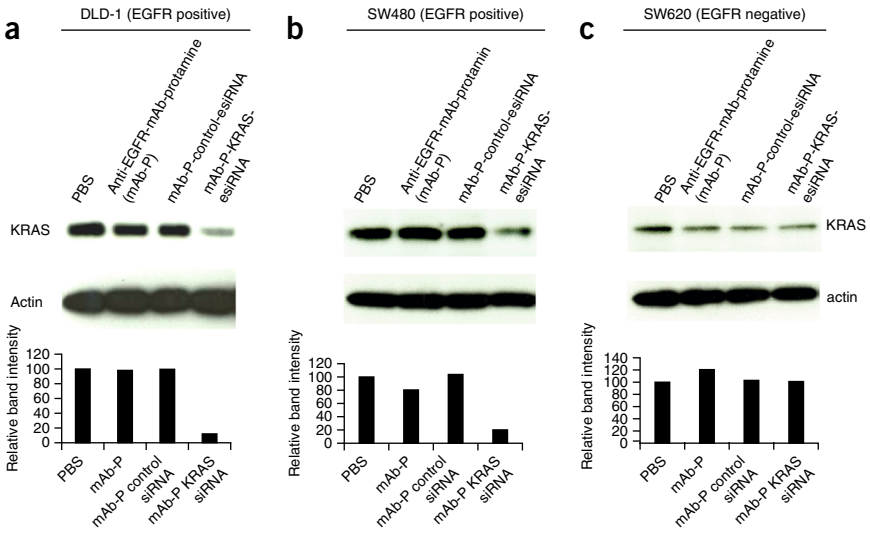

Figure 5 | mRNA target knockdown. (**a**,**b**) Anti-EGFR antibody–mediated RNAi reduces *KRAS* target gene expression in the EGFR-expressing carcinoma cell lines DLD-1 (**a**) and SW480 (**b**). (**c**) The EGFR-negative carcinoma cell line SW620 does not show a reduction in *KRAS* gene expression upon treatment with CSP-KRAS siRNA. DLD-1, SW480 and SW620 cells were exposed to 60 nM of anti-EGFR mAb-sulfo-SMCC–protamine (mAb-P) coupled to GFP control esiRNA (mAb-P-control-esiRNA) or to KRAS esiRNA (mAb-P-KRAS-esiRNA) for 72 h at 37 °C. Western blot analysis was performed for KRAS, with actin used as a loading control. Expression of KRAS was suppressed upon mAb-P-KRAS esiRNA treatment in EGFR-positive SW480 cells to 20% of control, and in DLD-1 cells to 15% of control, but not in EGFR-negative SW620 cells. Densitometry measurements (bar graphs) underline these observations.

expression systems can result in irreversible precipitation and the expression in mammalian cell systems can lead to reduced yield. These facts led to the development of rather complicated strategies, such as expressed protein ligation by oxidative disulfide bond formation between the targeting fragment and siRNA carrier fragment[21]. In addition, in our hands, preliminary experiments had several intrinsic shortcomings, including problems related to refolding to tertiary structure, expected yield and so on. The expression of genetically engineered delivery fragments offers enhanced technical and scientific flexibility, but the procedure presented here has the advantage of using readily available and clinically used purified targeting antibodies.

Consequently, our method provides a fast and reliable way to yield stable and cell type–specific transfer carriers for siRNA[1] by a receptor-dependent, antibody-mediated delivery system that is applicable to *in vitro* and *in vivo* experiments.

Experimental design

To target a certain cancer cell, one has to look for a surface marker and a specific antibody or antibody derivative that binds to this surface protein and provokes the internalization of the antigen-antibody complex into the cell, which in our example is the anti-EGFR-antibody cetuximab to treat colon cancer cells. Next, the antibody needs to be loaded with the (e)siRNA[13,22] against an oncogene of interest. Here we coupled the EGFR-specific antibody to the highly cationic charged peptide protamine by the use of the bispecific cross-linker sulfo-SMCC. The anionic charged siRNA binds to the protamine part of the complex by means of electrostatic interaction. As the highly charged cargo of the antibody might influence receptor detection and internalization, this process was tested by flow cytometry. The application of the complex is quite simple: cells can be incubated with it in culture, colony assays in semi-solid medium can be treated repeatedly by pipetting the complexes onto the medium, and the complex can be systemically injected into mice, leading to the inhibition of tumor growth in anti-EGFR–KRAS–siRNA–treated cell lines, such as DLD-1, SW480, HCT116 and LoVo[1]. The stability of the complex against enzymatic digest also allows a repeated treatment of cell cultures during cell growth.

Relevant controls

As RNAi controls, esiRNA delivery experiments by cetuximab were controlled by the application of control esiRNA raised against Xenogen GFP, and thus they had no influence on the expression of KRAS or other cellular gene products[8].

In western blot knockdown analysis, besides control esiRNA, active KRAS esiRNA and a PBS control, another sample is treated with the antibody-protamine conjugate with no esiRNA for exhibition of antibody-dependent effects. Off-target effects of the

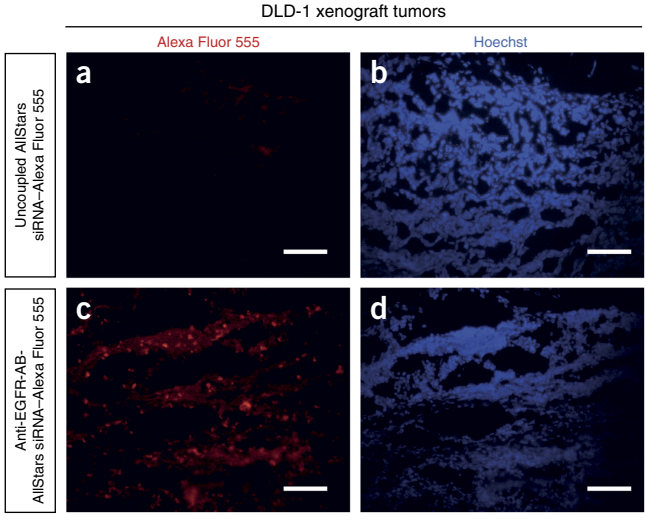

Figure 6 | The presence of antibody-siRNA complexes in xenograft-transplanted cells *in vivo* after intraperitoneal injection. A total of 1×10^7 DLD-1 colon adenocarcinoma cells were subcutaneously implanted in CD1 nude mice. (**a**–**d**) After reaching an average tumor size of 200 mm^3, mice were injected intraperitoneally with 4 mg/kg anti-EGFR mAb–Alexa Fluor 555–labeled AllStars negative control siRNA or with the uncoupled negative control siRNA. Tumors were resected 15 h after injection. In mice injected with mAb-555-siRNA, cryosections of tumors showed Alexa Fluor 555 fluorescence signals in the tumor rim (**c**). (**b**,**d**) Depiction of the same sections as in **a** and **c**, stained with Hoechst showing nuclei. (**a**) In mice injected with uncoupled siRNA–Alexa Fluor 555, no fluorescence signals were detectable. Scale bar, 100 mm. Animal experiments were strictly performed in compliance with local animal experiment permission no. 84.02.04.2014.A285, dated 20 October 2014, of the institutional animal care and use committee LANUV NRW.

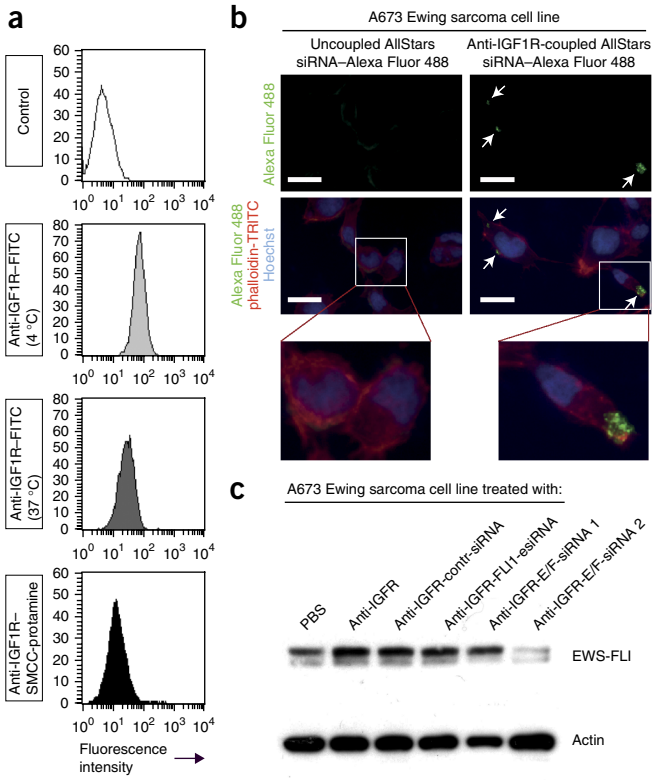

Figure 7 | Antibody–siRNA complex formation can be applied to IGF1R targeting. (**a**) Shown here by flow cytometry, A673 Ewing sarcoma cells internalize anti-IGF1R antibody GR11L–sulfo-SMCC–protamine complexes at 37 °C, such as the uncoupled GR11L antibody, which is depicted by a leftward shift in histogram signal compared to the non-internalized 4 °C control. (**b**) Green fluorescent cytoplasmic vesicular structures in A673 cells consisting of Alexa Fluor 488–siRNA were internalized by GR11L-protamine (arrows, right images), but not in the control experiment lacking the antibody conjugate (left images). Internalized Alexa Fluor 488–siRNA can be seen as green vesicular deposits (white arrows). Counterstaining of cell nucleus by Hoechst is shown in blue, and staining of the cytoskeleton by phalloidin-TRITC is shown in red. Boxed areas illustrate higher magnifications of the cells indicated. Scale bars, 20 µm. (**c**) The GSP complex was then coupled to siRNA against the mRNA of the oncogenic fusion protein EWS-FLI1, and A673 cells were treated with these complexes. As a result, EWS-FLI1 expression was downregulated as detected here in a western blot of FLI1 expression. EWS-Fli1 (E/F)- specific siRNA 2 (see Reagents for sequence information) reduced EWS-FLI1 protein expression by 80% compared to control siRNAs and the PBS control. Other E/F-specific siRNAs (siRNA 1 and FLI1-esiRNA) proved to be much less effective. EWS-FLI1 traveled as a double band at ~64 kDa, actin at 43 kDa. This alternative protocol to form and use the GSP-siRNA complex is described in **Box 1**.

targeting antibody can be evaluated by using cells that are negative for the targeted receptor—e.g., EGFR-negative SW620 colon cancer cells for the EGFR-targeted procedure (**Fig. 5**) originating from the same patient as the EGFR-positive SW480 cells.

Limitations

The principle of advanced chemical cross-linking tightly depends on the availability and accessibility of the corresponding amino acid residues. For instance, if the availability of reduced cysteine residues in the antibody to be coupled on the sulfo-terminal side of the cross-linker is diminished because of oxidation processes to disulfide bonds, the coupling efficiency will be low. This situation can be counteracted with the prior application of reducing agents to increase the number of accessible cysteines. Please keep in mind that the efficiency of reducing agents is dependent of the intrinsic redox state of the corresponding protein, and also that over-reduction may lead to loss of biological activity.

MATERIALS

REAGENTS

- A673 human Ewing sarcoma cell line (Sigma-Aldrich, cat. no. 85111504) **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and not infected with mycoplasma.
- AllStars negative control siRNA with Alexa Fluor 488 (Qiagen, cat. no. 1027284)
- AllStars negative control siRNA with Alexa Fluor 555 (Qiagen, cat. no. 1027286)
- AllStars negative control siRNA (Qiagen, cat. no. 1027281)
- Anti–β-actin mAb (clone AC-15, Sigma-Aldrich)
- Anti-FLI1 C-19 rabbit polyclonal antibody (Santa Cruz Biotechnology, cat. no. sc-356)
- Anti-KRAS antibody (ab55391, Abcam)
- BSA standard, 2 mg/ml (Thermo Scientific, cat. no. 23209)
- Cetuximab (Erbitux 5 mg/ml, Merck Serono)
- CD-1 nude mouse (NU-Foxn1nu, Charles River, strain code 087) **! CAUTION** All animal experiments shown here were strictly performed in compliance with local animal experiment permission no. 84.02.04.2014. A285, dated 20 October 2014, of the institutional animal care and use committee 'Landesamt fuer Natur, Umwelt und Verbraucherschutz NRW' (LANUV NRW). Take care to follow appropriate institutional regulatory board guidelines before performing any animal experiments.
- DAKO fluorescent mounting medium (DAKO, cat. no. 3023)
- Difco agar, Noble (Becton Dickinson, cat. no. 214220)
- DLD-1 human colon adenocarcinoma cell line (Sigma-Aldrich, cat. no. 90102540) **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and not infected with mycoplasma.
- EGFR-specific antibody (528), FITC (Santa Cruz Biotechnology, cat. no. sc-120-FITC)
- esiRNA, FLI1 HU-09196-1 (Eupheria)
- esiRNA, KRAS EHU-11443-1 (Eupheria)
- esiRNA, GFP control EHU-GFP (Eupheria)
- Ethidium bromide solution, 10 mg/ml (Roth, cat. no. 2218.2) **! CAUTION** This solution is harmful if inhaled, and it is suspected of causing genetic defects. Wear protective gloves, lab coat and eye protection during use.
- EWS/FLI1-siRNA1 (Dharmacon, sense: 5′-GGCAGCAGAACCCUUCUU AUU-3′; antisense: 5′-UAAGAAGGGUUCUGCUGCCUU-3′)
- EWS/FLI1-siRNA2 (Dharmacon, sense: 5′-GCAGAACCCUUCUUAUGA CUUUU-3′; antisense: 5′-AAGUCAUAAGAAGGGUUCUGCUU-3′)
- FBS (Biochrom, cat. no. 90115K)
- Hoechst 33342 (Life Technologies, cat. no. H3570)
- Anti-IGF1R (Ab-1) mouse mAb (aIR3; Calbiochem, cat. no. GR11L)
- Iodonitrotetrazolium chloride (INT; Sigma-Aldrich, cat. no. I8377)
- L-Glutamine, 200 mM (Invitrogen, cat. no. 25030024)
- Novex Tris-borate-EDTA (TBE) running buffer, 5× (Invitrogen, cat. no. LC66)
- Paraformaldehyde (Sigma-Aldrich, cat. no. P6148), **! CAUTION** Paraformaldehyde is toxic; avoid inhalation and contact with skin and eyes.
- PBS, Dulbecco's (Sigma-Aldrich, cat. no. D8537)
- PBS, Dulbecco's powder (Merck, cat. no. L182-50)
- Phycoerythrin (PE)-goat anti-mouse Ig (secondary antibody; BD Pharmingen, cat. no. 550589)

Box 1 | Targeting IGF1R-positive cells with GSP complex (GR11L-sulfo-SMCC-protamine)

Forming the GSP complex ● TIMING 2 d

1. Combine 30 µl of 20 mg/ml protamine solution, 140 µl of 10 mg/ml sulfo-SMCC solution and 370 µl of ddH_2O, and incubate the mixture for a minimum of 1 h, with shaking at 700 r.p.m. in a Thermomixer at 37 °C.
2. Add 1 ml of GR11L to sulfo-SMCC-protamine sulfate and incubate it overnight at 4 °C.

! CAUTION Do not desalt GR11L before coupling; GR11L is stored in PBS and it has a low concentration and will be less after desalting, and therefore leave out desalting before coupling.

3. The next day, desalt GSP sulfate, as described in step 10.
4. After desalting, measure the protein content via BCA assay, as described in steps 11–13. The concentration will be ~1.23 µM. The next steps (**Fig. 7**) are calculated using this concentration.

Assessment of antibody functionality of the GSP complex via flow cytometry ● TIMING 7–8 h

5. Use 5 × 10^5 A673 human Ewing sarcoma cells per condition. Detach the cells with trypsin as follows: Remove the medium from the cells growing in a 75-cm^2 flask, wash the cells once with 10 ml of PBS, remove PBS, add 2 ml of trypsin to the cells and incubate the cells at 37 °C until you observe detachment of the cells—i.e., by light microscopy. Suspend cells in 8 ml of RPMI medium to stop the reaction. Count the cells and pipette 5 × 10^5 cells into each FACS tube. Prepare four FACS tubes for the following conditions.

Tube	GR11L (60 nM)	GSP (60 nM)	PBS	GAM (secondary antibody goat anti-mouse)
1. Unstained	–	–	100 µl	–
2. Positive control 4 °C	4.9 µl plus 95.1 µl PBS 1 h, 4 °C	–	–	2 µl
3. Cells treated with GR11L	4.9 µl plus 95.1 µl PBS 3 h, 37 °C		–	2 µl
4. Cells treated with GSP		4.9 µl plus 95.1 µl PBS 3 h, 37 °C	–	2 µl

6. Add 1 ml of PBS to the cells in each tube, and vortex briefly and centrifuge the tubes for 5 min at 400*g*. Decant the supernatant.
7. For 60 nM GR11L and GSP in tubes 3 and 4, add 4.9 µl to 95.1 µl of PBS and incubate the tubes for 3 h at 37 °C. For the positive control in tube 2, add 4.9 µl of 60 nM GR11L in 95.1 µl of PBS and incubate the tube for 1 h at 4 °C.
8. Add 100 µl of PBS to tube 1 and store it on ice during incubation time.
9. Add 1 ml of PBS to the cells, vortex briefly and centrifuge the cells for 5 min at 400*g*. Decant the supernatant.
10. Next, add 2 µl of PE-labeled goat anti-mouse secondary antibody to tubes 2, 3 and 4. Incubate the tubes for 1 h at 4 °C.
11. Add 1 ml of PBS to the cells, vortex briefly and centrifuge them for 5 min at 400*g*. Decant the supernatant.
12. Add 200 µl of fresh PBS to the cells. Vortex and analyze internalization reaction via FACS.

Coupling of siRNA to GSP ● TIMING 2–5 d

13. Seed 2 × 10^4 A673 human Ewing sarcoma cells per well and condition on a 12-well plate. Fill up each well with RPMI medium to 1.5 ml. Incubate overnight at 37 °C.
14. Couple siRNA to GSP in a ratio of 5:1. For one well with an end concentration of 60 nM GR11L and 300 nM siRNA in 750 µl of medium, add 15 µl of 15 µM siRNA to 36.6 µl of 1.23 µM GSP from Step 3 of this box and incubate it for 2 h at RT.
15. Remove the medium from the cells. Refill with 750 µl of fresh medium and add 51.6 µl of the GSP–siRNA complex according to the different experimental conditions: for instance, PBS control, noncoupled CSP, CSP–control siRNA and CSP–KRAS siRNA.
16. Incubate the cells overnight with GSP–siRNA complexes.
17. The next day, repeat the treatment as described in steps 14–16 of this box.
18. Perform treatment a total of three or four times.
19. After last treatment, detach cells with trypsin, as described in step 5 of this box, and use them directly for the respective assays described in Step 27 of the PROCEDURE.

- Penicillin-streptomycin (Sigma-Aldrich, cat. no. P0781)
- Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, cat. no. 111-036-045)
- Peroxidase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, cat. no. 115-036-062)
- Phalloidin-tetramethylrhodamine B isothiocyanate (phalloidin-TRITC; Sigma-Aldrich, cat. no. P1951)
- Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, cat. no. 23225)
- Protamine sulfate salmon milt (Calbiochem, cat. no. 539122)
- RPMI 1640 medium (Sigma-Aldrich, cat. no. R8758)
- Sulfo-SMCC, no-weigh format (Thermo Scientific, prod. no. 22622)
- SW480 human colon adenocarcinoma cell line (Sigma-Aldrich, cat. no. 8709280) **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and not infected with mycoplasma.
- SW620 human colon adenocarcinoma cell line (Sigma-Aldrich, cat. no. 87051203) **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and not infected with mycoplasma.
- Trypsin (Cambrex Bio Science)

EQUIPMENT
- Bio-Rad cell counter TC20 (Bio-Rad)
- Bio-Rad counting slides for cell counter (Bio-Rad)
- Centrifuge, Sigma 4K15 (Sigma-Aldrich)
- Coverslips, 24 × 50 mm (Engelbrecht, cat. no. k12450)
- Eppendorf Thermomixer comfort (Eppendorf)
- FACSCalibur flow cytometer (Becton Dickinson)
- Greiner CELLSTAR six-well culture plates (Sigma-Aldrich, cat. no. M8562)
- Greiner CELLSTAR 96-well plates (Sigma-Aldrich, cat. no. M0812)
- Incubator, Heracell 150i (Thermo Scientific)
- Laminar flow, Kendro Herasafe 18 (Heraeus)
- NanoDrop ND-1000 spectrophotometer (PEQLAB)
- Nunc Lab-Tek II chamber slide, four wells (Thermo Scientific, cat. no. 154526)
- Reaction tubes, 1.5 ml (Eppendorf)
- Serological pipettes 5, 10 and 25 ml, Costar Stripette (Corning)
- Tubes for flow cytometry (Sarstedt, cat. no. 55.1579)
- Zeiss Axioskop II with Axiocam MRc
- Zeba spin desalt columns for gel filtration (Thermo Scientific, prod. no. 89892)
- Zeiss Axio Vert.A1 fluorescence microscope (Carl Zeiss)

REAGENT SETUP

Ethidium bromide solution Add 20 µl of ethidium bromide to 200 ml of H_2O. Use the solution for a maximum of 1 week. Store it at room temperature (RT, 21 °C) in the dark.

Hoechst, 1 mg/ml Add 10 mg of Hoechst 33342 to 10 ml of ddH_2O. Store it at 4 °C for up to 1 year.

Iodonitrotetrazolium chloride (INT), 10× staining solution Prepare a 1 mg/ml staining solution by adding 50 mg of INT to 50 ml of ddH_2O. Store it at 4 °C in the dark for a maximum of half a year.

PBS, 10× Add 9.55 mg of PBS to 100 ml of ddH_2O. Stir the mixture until everything is dissolved. Store it at RT for a year.

Paraformaldehyde (PFA), 4× For 4% (wt/vol) PFA solution, add 20 g of paraformaldehyde to 450 ml of ddH_2O. Afterward, add five drops of 1 N NaOH. Stir the PFA solution on a heat block at 50 °C until everything is dissolved. Next, add 50 ml of 10× PBS. Fill the solution up to 500 ml, divide it into aliquots in 50-ml tubes, and store them for up to 1 year at −20 °C until use.

Protamine solution, 10 mg/ml Dissolve protamine sulfate at a concentration of 10 mg/ml in ddH_2O. Add 100 mg to 10 ml of ddH_2O. If necessary, warm up the solution to 40 °C to dissolve completely. Divide the solution into aliquots and store them at −20 °C until use. This solution can be stored for 1 year. Be sure to re-dissolve frozen protamine sulfate solution completely after thawing.

Protamine solution, 20 mg/ml Dissolve protamine sulfate at a concentration of 20 mg/ml in ddH_2O. Add 200 mg to in 10 ml of ddH_2O. If necessary, warm the solution to 40 °C to dissolve it completely. Divide the solution into aliquots and store them at −20 °C until use. This solution can be stored for 1 year. Be sure to re-dissolve frozen protamine sulfate solution completely after thawing.

RPMI medium Add 10% (vol/vol) FBS, 1% (vol/vol) penicillin-streptomycin and 1% (vol/vol) L-glutamine to 500 ml of RPMI 1640. Shake well and store the medium at 4 °C. Use it for a maximum of 4 weeks. Prewarm at 37 °C before use.

Soft agar, 3% (wt/vol) Add 3 g of Difco agar noble to 100 ml of ddH_2O. Boil the mixture for 1 min to dissolve the agar. Repeat boiling until the agar is dissolved completely. Fill 5 ml at a time in a 15-ml Falcon tube as long as the soft agar is in liquid form. Let it stand for a few minutes to cool down. Store the agar until use at −20 °C for up to 1 year.

Sulfo-SMCC solution Freshly prepare a 10 mg/ml sulfo-SMCC solution by dissolving 2 mg of no-weight-format sulfo-SMCC in 200 µl of water. Shake for ~2–3 h at 37 °C to dissolve. Use it on the same day.

Phalloidin–tetramethylrhodamine B isothiocyanate (TRITC), 1:1,000 Add 1 µl of phalloidin-TRITC to 999 µl of ddH_2O. Keep it on ice until use. Always freshly prepare the solution.

PROCEDURE

Coupling of sulfo-SMCC to protamine ● TIMING 3–4 h

1| Combine 50 µl of 10 mg/ml sulfo-SMCC solution (equaling 50 mM starting concentration) with 500 µl of 10 mg/ml protamine solution (equaling 3 mM starting concentration) dissolved in water. Protamine sulfate comprises a mixture of peptides averaging 8 kDa in molecular weight. This makes an average molecular ratio of 1.7:1 of sulfo-SMCC to protamine in the respective solution of 550 µl with calculated effective end concentration of 1.25 mM sulfo-SMCC–activated protamine.

2| Incubate the protamine–sulfo-SMCC mixture for 2 h by shaking it at 500 r.p.m. at RT.

? TROUBLESHOOTING

■ **PAUSE POINT** You can extend this incubation time up to 5 h at 4 °C or decrease it by incubating it for 1 h at 37 °C.

Desalting cetuximab before use ● TIMING 1 h

3| To desalt the mAb cetuximab before use, take a Zeba spin desalting column, remove the column's bottom closure, transfer it to a 15-ml collection tube and centrifuge it for 2 min at 1,000*g* at RT to exchange the storage solution. Discard the flow-through.

4| To equilibrate the column with cell culture–grade PBS, add 2.5 ml of PBS to the spin column and centrifuge it again for 2 min at 1,000*g* and RT. Discard the flow-through.

5| Repeat Step 4 three additional times.

6| Before adding cetuximab, centrifuge the column again for 2 min at 1,000*g* at RT to remove remaining PBS.

7| Load 2.2 ml of cetuximab on the column. Place the desalting column in a new collection tube and centrifuge it for 2 min at 1,000*g* at RT, collecting the flow-through containing the cetuximab. The cetuximab is now ready to use.

Coupling of cetuximab to sulfo-SMCC–protamine ● TIMING 1 d

8| Add the desalted cetuximab in a molecular excess of 23:1 to the sulfo-SMCC–protamine conjugate. For instance, add 2.2 ml of cetuximab solution (equaling 31 μM concentration) to the complete activated 550 μl of sulfo-SMCC–protamine solution from Step 2 (equaling 2.7 mM concentration), resulting in effective end concentrations of 23 μM cetuximab and 540 μM activated protamine in 2,750 μl.

? TROUBLESHOOTING

9| Mix and incubate overnight at 4 °C without shaking.

Desalting cetuximab–sulfo-SMCC–protamine ● TIMING 1 h

10| To desalt cetuximab–sulfo-SMCC–protamine (CSP) again after coupling, equilibrate a Zeba spin desalting column as described in Steps 4–6 and load the CSP conjugate from Step 9 onto the column.

▲ **CRITICAL STEP** Do not load more than 2.5 ml on one column; if necessary, use two columns for desalting.

■ **PAUSE POINT** In this form, CSP can be used and stored at 4 °C for several months.

Measurement of protein content via Pierce bicinchoninic acid protein assay ● TIMING 2 h

11| Prepare 5 ml of bicinchoninic acid (BCA) solution by combining reagent A and reagent B from the Pierce BCA assay kit in a ratio of 50:1; add 100 μl of reagent B to 4.9 ml of reagent A. This provides enough BCA solution for a standard curve, one blank sample and your CSP sample.

12| Use a 2 mg/ml BSA standard to make dilutions for the standard curve as described in the table below. Vortex and incubate the probes for 30 min at 37 °C.

■ **PAUSE POINT** The incubation time can be extended to up to 1 h.

Sample	BCA solution (μl)	BSA standard, 2 mg/ml (μl)	CSP (μl)	ddH_2O (μl)
Standard 4 mg/ml	400	10	–	–
Standard 2 mg/ml	400	5	–	5
Standard 1 mg/ml	400	2.5	–	7.5
Standard 0.5 mg/ml	400	1.25	–	8.75
Standard 0.25 mg/ml	400	0.625	–	9.375
Blank	400	–	–	10
CSP sample	400	–	5	5

13| Measure the protein content via NanoDrop at an optical density at 600 nm (OD_{600}), and convert the protein concentration (mg/ml) to molarity (μM). Usually, the concentration of CSP is ~23 μM.

Assessment of antibody functionality via flow cytometry ● TIMING 5 h

14| Use 5 × 10^5 adherent EGFR-positive cells, such as DLD-1 or SW480 cells, for each of the following tubes. Detach the cells with trypsin as follows: remove the medium from the cells growing in a 75 cm^2 flask, wash the cells once with 10 ml of PBS, remove PBS, add 2 ml of trypsin to the cells and incubate the cells at 37 °C until you observe detachment of the cells—i.e., by light microscopy. Suspend cells in 8 ml of RPMI medium to stop the reaction. Count the cells and pipette 5 × 10^5 cells into each FACS tube. Prepare four FACS tubes for the following conditions:

Tube	Number of cells	Cetuximab	CSP
1. Unstained	5 × 10^5	–	–
2. Positive control	5 × 10^5	–	–
3. Cetuximab-treated cells	5 × 10^5	Cetuximab, 0.97 μl (31 μM) plus 500 μl of PBS	–
4. CSP-treated cells	5 × 10^5	–	CSP, 1.3 μl (23 μM) plus 500 μl of PBS

15| Before antibody treatment of the cells, add 1 ml of PBS to each tube, vortex the tubes briefly and centrifuge at 400*g* for 5 min at RT.

16| Discard the supernatant by decanting.

17| Repeat Steps 15 and 16.

18| Add uncoupled cetuximab to a final concentration of 60 nM (which means 0.97 µl of a 31 µM cetuximab solution) in 500 µl of PBS to the cells in tube 3 and 1.3 µl of a 23 µM CSP solution (final concentration of 60 nM of CSP) in 500 µl of PBS to the cells in tube 4. Vortex the tubes briefly and incubate them for 1 h at RT. The EGFRs should now be internalized into the cells.
▲ **CRITICAL STEP** During incubation of tubes 3 and 4, tubes 1 and 2 can be stored on ice.

19| Wash the cells in tubes 3 and 4 as described in Steps 15 and 16.

20| By binding to specific extracellular EGFR domains, cetuximab drives the majority of receptors to internalization. To detect the remaining surface-bound EGFR, use the EGF-specific antibody (528)-FITC, which binds to a different epitope than cetuximab. Add 20 µl of anti-EGFR–FITC antibody in 100 µl of PBS to the cells in tubes 2, 3 and 4, and then vortex the tubes and incubate them for 1 h on ice.

21| Add 1 ml of PBS to wash the cells in tubes 2, 3 and 4; vortex them briefly, centrifuge them at 400*g* for 5 min at RT and discard the supernatant.

22| Repeat Steps 15 and 16.

23| To analyze the cells, add 200 µl of fresh PBS and vortex the tubes.

24| Analyze the EGFR surface expression by FACS. Gate out dead cells and analyze the living cells according to their FITC signals.
? TROUBLESHOOTING

Coupling of CSP to siRNA ● TIMING 3 h–1 d
25| Couple CSP to the specific siRNA by adding them together in a 1:5 molecular excess and incubate CSP–siRNA for 2 h with shaking at 1,000 r.p.m. in a Thermomixer at RT: the concentration of CSP after desalting is 23 µM; all indicated siRNAs have a concentration of 15 µM. Use as an end concentration 60 nM of cetuximab and 300 nM of AllStars negative control siRNA, effector siRNA or esiRNA, respectively, on the cells. For example, to prepare cell lysates for western blotting in a six-well plate in 1.5 ml of RPMI medium as in Step 27, you will need 3.91 µl of CSP for an end concentration of 60 nM on the cells and 30 µl of siRNA for an end concentration of 300 nM on the cells.
! CAUTION Do not freeze/thaw siRNA. Store it frozen in single-use aliquots.
! CAUTION Use CSP–siRNA directly after incubation. Always freshly prepare CSP–siRNA and store the siRNA at –20 °C.
■ **PAUSE POINT** The incubation time can be extended to up to 12 h or overnight if stored at 4 °C. However, we have observed better results using 2 h of incubation at RT.

CSP siRNA payload assessment
26| To check the ability of the CSP to bind and carry siRNA, perform gel shift assays for siRNA load estimation (option A), fluorescence microscopy for visualization of internalized complexes (option B) or serum stability assay (option C).
(A) Gel shift assays ● TIMING 4 h
(i) Dilute the CSP preparation sample from Step 10 to 1 µM with water. Prepare the tube to co-incubate CSP with 20 µM AllStars negative control siRNA with Alexa Fluor 488 in various ratios according to the following scheme:

Reagent	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
CSP 1 µM (µl)	19	10	5	2.5	1
Alexa Fluor 488–siRNA 20 µM (µl)	1	1	1	1	1
Water (add to 20 µl)	0	9	14	16.5	18
CSP:siRNA ratio (mol/mol)	1:1	1:2	1:4	1:8	1:20

(ii) Leave the coupling reaction to complete for 2 h, with shaking at 1,000 r.p.m. in a Thermomixer at RT.

(iii) Add 3 µl of gel loading dye and apply it to 1.5% (wt/vol) agarose gel in 1× Novex TBE running buffer. Run the 10-cm 0.6% (wt/vol) agarose gel at 90 volts for 45 min. Monitor electrophoresis by Alexa Fluor 488 fluorescence of unbound and CSP-complexed Alexa Fluor 488–siRNA by exposing the gel on a UV transillumination device.

(iv) After reaching suitable electrophoretic separation, stain the gel using an ethidium bromide bath for 20 min and expose it to UV transillumination followed by imaging. Typically, free siRNA will be complexed by CSP to saturation of siRNA load capacity leading to the accumulation of non-migratory CSP:siRNA in the gel pouch. The molecular ratio of appearance of free siRNA monomers depicts the maximum siRNA load capacity of the CSP preparation.

! CAUTION Perform imaging via ethidium bromide staining and not via Alexa Fluor 488–siRNA.

(B) Fluorescence microscopy ● TIMING 3 d

(i) Seed 4 × 10^4 adherent EGFR cells, such as DLD-1 or SW480 cells, per well on a chamber slide. Seed the cells in a total of four wells.

(ii) Add 700 µl of RPMI medium with 10% (vol/vol) FBS on top of each well, and incubate it overnight at 37 °C.

(iii) The next day, couple enough CSP to fluorescent Alexa Fluor 488–siRNA for two wells. As controls, use uncoupled fluorescent siRNA for the other two wells. Calculate the amounts to couple based on a volume of 400 µl of medium per well. For example, using 23 µM CSP and 20 µM Alexa Fluor 488–siRNA, for an end concentration of 60 nM CSP and 300 nM Alexa Fluor 488–siRNA on the cells in 400 µl of RPMI medium per well, add 6 µl of siRNA to 1.04 µl of CSP for each well needed and incubate for 2 h at RT.

(iv) After coupling, remove the medium from the cells and add 400 µl of fresh and prewarmed RPMI medium per well to the cells.

(v) Add 7.04 µl of CSP–siRNA directly to the cells and incubate them overnight at 37 °C.

(vi) *Fixation and staining of the cells*. By using an inverse fluorescence microscope with GFP (excitation 484 nm and emission 507 nm) filter set, estimate the internalization of Alexa Fluor 488–siRNA *in vivo* at 200× magnification. If you observe fluorescent siRNA-containing intracellular vesicles, fix and stain the cells as described in the following steps; if not, incubate the cells for an additional 4 h.

(vii) Fixation, counterstaining and mounting of slides should be carried out to allow maximal optical resolution by upright fluorescence microscopy, according to the following steps: before fixation, wash the cells carefully with PBS. Next, remove the medium and add ~500 µl of PBS to the cells. Shake carefully and remove the PBS without touching the cells.

(viii) Repeat Step 26B(vii) three additional times (a total of four washes).

(ix) Next, add 400 µl of 4% PFA and incubate the cells for 1 h at 4 °C.

■ PAUSE POINT Incubate the cells for at least 1 h. Incubation time can be extended up to overnight.

(x) Remove PFA solution and add ~500 µl of PBS to the cells. Shake it carefully and remove PBS without touching the cells. Repeat this washing step another three times (a total of four washes).

(xi) To stain actin cytoskeleton of the cells, add 320 µl of PBS per well and in addition 80 µl of a 1:1,000 phalloidin-TRITC dilution. Next, incubate the cells for 1 h at 4 °C.

(xii) Remove phalloidin-TRITC solution and add ~500 µl of PBS to the cells. Shake the mixture carefully and remove PBS without touching the cells. Repeat this washing step another three times (a total of four washes).

(xiii) To stain the nucleus of the cells, remove the medium and add 300 µl of PBS to the cells. In addition, add 10 µl of Hoechst at a concentration of 1 mg/ml. Shake the cells carefully and incubate them for 10 min at RT.

■ PAUSE POINT Incubation time can be extended to up to 1 h at RT.

(xiv) Remove the Hoechst solution and add ~500 µl of PBS to the cells. Shake the cells carefully and remove the PBS without touching the cells. Repeat this washing step another three times (a total of four washes).

(xv) *Mounting of slides for microscopic analysis*. To mount slides, remove all of the PBS and remove the chambers from the chamber slides according to the manufacturer's recommendations.

(xvi) Cover the cells with four drops of DAKO fluorescent mounting medium, and then mount the coverslip onto the slide.

(xvii) Store the slides at 4 °C in the dark for examining internalization of fluorescent siRNA under an upright fluorescence microscope equipped with green, red and blue fluorescence filter units. Microscopic analysis should reveal green fluorescent, cytoplasmic vesicles of internalized Alexa Fluor 488–siRNA, which are ideally present in a majority of cells. For better subcellular orientation, use Hoechst-stained nuclei (blue fluorescence filter unit, excitation 358 nm and emission 463 nm) and the phalloidin-TRITC (red fluorescence filter unit, excitation 554 nm and emission 581 nm).

(C) Serum stability assay ● TIMING 25 h

(i) Take the CSP preparation sample from Step 10. Co-incubate it with 20 µM AllStars negative control siRNA–Alexa Fluor 488 according to the following scheme: prepare and label eight tubes with 13 µl of water, 3 µl of the CSP preparation (23 µM) and 2 µl of Alexa Fluor 488–siRNA each. Leave them to react for 2 h at RT.

(ii) Add 2 µl of FBS to tube 8, 24 h before running the gel. At the same time, add 2 µl of water to the control tube 7.

(iii) Add 2 µl of FBS to tube 6, 6 h before running the gel, and 2 µl of water to control tube 5.
(iv) Prepare tubes 3 and 4 as in Step 26C(iii), 2 h before running the gel.
(v) Prepare tubes 1 and 2 as in Step 26C(iii) 1 h before running the gel.
(vi) Supplement all eight tubes with 3.3 µl of 6× gel loading dye and completely apply them to a 0.6% (wt/vol) agarose gel.
(vii) Run the gel at 90 volts for 2 h, stain it with ethidium bromide, and expose it to the imaging unit.

Downstream application of CSP–siRNA complexes

27| After CSP–siRNA coupling, the complex can be used for different assays *in vitro* and *in vivo*. Use option A for the treatment of cells to obtain protein lysates for western blot analysis or other assays (BrdU incorporation, annexin V staining), option B for the colony forming assay (soft agar) to test for anchorage-independent tumor cell growth, or option C for targeting and analyzing colorectal cancer (CRC) cell lines in a xenograft model *in vivo*.

(A) Treatment of cells to obtain protein lysates for western blotting or other assays ● TIMING 2–5 d

(i) Use a six-well-plate format for treatments of DLD-1, SW480 or SW620 cells.
(ii) Seed 1 × 10^5 cells per condition and per well in 3 ml of medium and incubate it overnight.
(iii) The next day, couple CSP to siRNA for a minimum of 2 h at RT (see Step 25). Use a molar ratio of 1:5 of cetuximab to siRNA, as described in Step 25.
(iv) Remove the medium and add 1.5 ml of fresh medium.
(v) Add the CSP–siRNA complex to the cells to a final concentration of 60 nM cetuximab, and then incubate the cells overnight at 37 °C.
(vi) The next day, couple CSP to siRNA again for a minimum of 2 h, and then remove the medium with the treatment solution and add 1.5 ml of fresh medium. Add the newly coupled CSP–siRNA to the well and continue with the next treatment the next day.
(vii) If needed, repeat Step 27A(vi) for an additional one or two times in the next days, leading to three or four consecutive treatments for optimal results.
! CAUTION Check the cells after and before each treatment if the treatment shows an effect. If cells already start to detach from the plate, or if they start to look unhealthy and apoptotic, stop the treatment and start your assays directly or prepare lysates.
■ PAUSE POINT Repetitions of treatments can be adjusted depending on the condition of the cells.
(viii) After the last treatment, detach the cells with trypsin and resuspend them in medium as described in Step 14 and use them directly for further assays such as annexin V staining[1] to detect the effect of siRNA treatment on apoptosis or BrdU incorporation[1] to determine the effect of siRNA treatment on the proliferation of cells. Alternatively, use the cells to obtain lysates for western blotting[1] to analyze downstream target gene expression after siRNA treatment.

(B) Colony-forming assay (soft agar) to test for anchorage-independent tumor cell growth ● TIMING 7–8 h (incubation time 1–2 weeks)

(i) Use any adherent EGFR-positive cell line of your choice. Here we describe the assay using SW480 cells. Trypsinize as described in Step 14 and count the cells. We advise seeding two different cell numbers—for example, a group of 3,000 cells per well and a second of 5,000 cells per well—as technical triplicates.
(ii) Prepare the 3% (wt/vol) soft agar as described in Reagent Setup, and then boil the solid agar in a microwave before use in Step 27B(vi), so that the solid agar becomes soft and sterile. This step can be repeated if the agar starts to get solid again during the procedure. Let the agar cool down for 2–3 min by allowing it to stand at RT.
(iii) For one condition in triplicate with 3,000 cells per well, add 12,000 cells to one reaction tube and fill it up with medium to a total volume of 120 µl.
! CAUTION Make calculations for four wells to compensate for the loss of volume because of the viscous agar. Therefore, take 12,000 cells to treat instead of 9,000.
(iv) To pretreat cells with CSP–siRNA complexes before seeding them, couple a final concentration of 60 nM CSP to 300 nM siRNA (see also Step 18). For 120 µl of cells, add 0.313 µl of CSP to 2.4 µl of siRNA and incubate for 2 h with shaking at 700 r.p.m. in a Thermomixer at RT.
(v) After coupling, add CSP–siRNA to the cells and resuspend them well.
(vi) Incubate the cells for a minimum of 1 h with CSP–siRNA.
▲ CRITICAL STEP This step enhances the efficiency of the treatment.
■ PAUSE POINT You can extend this incubation time to up to 3–4 h.
(vii) Add 168 µl of the prewarmed 3% (wt/vol) soft agar from Step 27B(ii) and 432 µl of medium to the treated 120 µl of cells. Resuspend the cells well by pipetting without causing air bubbles.
(viii) Add 180 µl of the suspension per well into three wells.
! CAUTION Prevent air bubbles from forming in the soft agar.

(ix) Leave the plate for a few minutes at RT so that the agar can solidify.
(x) Incubate the 96-well plate at 37 °C, 5% CO_2 and high humidity.
(xi) One day after plating, add 50 µl of RPMI medium to each well to prevent the agar from drying out. Change the top medium every 3–4 d to feed the cells.
(xii) Perform treatment in culture with the CSP–siRNA complex 1 week after seeding. Calculate a final concentration of 60 nM CSP–siRNA complex to the total volume of 230 µl per well (180 µl of cell suspension with agar plus 50 µl of medium on top). Take 0.6 µl of a 23 µM CSP solution plus 6 µl of a 15 µM siRNA solution and incubate it for 2 h, as described in Step 25. Add the treatment solution directly on top of each well and incubate it at 37 °C. Change the top medium (without CSP–siRNA) every 3–4 d to feed the cells.
(xiii) *Fixing and staining of cells in a colony-forming assay*. After 1–3 weeks, depending on the colony size, the colonies can be fixed and stained. To fix the cells, remove the medium from the agar and add 18 µl of 10× INT staining solution.
(xiv) Incubate the plate overnight at 37 °C in the dark.
(xv) Determine the number of colonies using a binary microscope.

(C) Targeting and analyzing CRC cell lines in a xenograft model *in vivo* ● TIMING 7–8 h (incubation time 4–8 weeks)

(i) For the transplantation of CD1 nude mice, cultivate DLD-1 or SW480 cells. Calculate 1 × 10^7 cells per xenotransplanted tumor. Detach the cells by trypsinization as described in Step 14 and collect them in full RPMI medium.
! CAUTION All animal experiments shown here were strictly performed in compliance with local animal experiment permission no. 84.02.04.2014.A285, dated 20 October 2014, of the institutional animal care and use committee LANUV NRW. Take care to follow appropriate institutional regulatory board guidelines before performing any animal experiments.
(ii) Collect the cells by centrifugation (for 5 min at 400*g* at RT) and wash them twice with PBS; resuspend them at a concentration of 6.67 × 10^7 cells per ml in PBS.
(iii) Transplant mice subcutaneously in one flank with 150 µl of the cell suspension now containing 1 × 10^7 cells. Use eight mice in the treatment group and eight in each control group (CSP-only control group and CSP-control siRNA group, using for example esiRNA GFP-control) to result in significant tumor size differences.
(iv) Supervise mouse and tumor growth. Typically, after 7–10 d, tumors reach notable sizes of 100 mm^3 or more, and they can be treated by applications of CSP–siRNA.
(v) Preparation of CSP–siRNA for *in vivo* treatment. Prepare CSP–(e)siRNA as described in Step 25. For a single dose, calculate 4 mg of cetuximab per kg mouse.
(vi) Determine the weight of each tumor-bearing recipient mouse. Inject 4 mg/kg mouse CSP–(e)siRNA intraperitoneally[23] in a maximum of 150 µl of injection volume per treatment. To obtain the results in **Figure 4**, we coupled Alexa Fluor 555 siRNA (AllStars negative control siRNA–Alexa Fluor 555) to CSP and injected one single dose. For reduction of tumor size, treat mice three times a week for ~3 weeks until tumor sizes of larger than 1,500 mm^3 in control groups require mouse euthanasia and tumor preparation according to your local animal welfare specifications. Determine the tumor size by caliper measurements twice a week, and calculate tumor volumes by the formula length × $width^2$ × 0.52 (see Schmidt *et al.*[24]).
(vii) Euthanize mice for sample preparation by deep anesthesia via isoflurane inhalation followed by cervical dislocation[25]. Embed tumor samples according to in-house methods to perform paraffin and/or frozen sectioning[26]. Tumor histology can be analyzed after standard H&E staining[27]. To determine the number of proliferating tumor cells, stain sections for expression of the proliferation marker Ki67 (ref. 1). The incorporation of Alexa Fluor 555–siRNA can be visualized using a fluorescence microscope after cryosectioning and washing of the sections twice for 5 min with PBS, once for 5 min with Hoechst 33342 stock solution diluted 1:1,000 in PBS, and twice with PBS for 5 min. Mount the coverslips onto the slides, as described in Step 26B(xvi).
(viii) Test the significance of changes in tumor volume measurements by comparing groups by paired, two-sided Student's *t*-test.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Precipitation reaction occurs during the protamine coupling reaction	Concentration of the sulfo-SMCC–protamine mix is too high	Dilute the sulfo-SMCC–protamine coupling mix with ddH_2O. Start with a few microliters. Agitate the mixture, and stop when the precipitates are dissolved

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
8	Precipitation reaction occurs during the siRNA coupling reaction	The binding of anionic siRNA and cationic conjugate leads to charge extinction and loss of solubility	Agitate the mixture at 25 °C with 800 r.p.m. Usually, the precipitate dissolves after several minutes. If not, dilute it with PBS until clear
24	No internalization reaction after treating the cells for 1 h with cetuximab	EGFR-expressing cell lines differ in their internalization rates and times	Increase the incubation time with cetuximab at Step 18 to up to 12 h or overnight. Some cell lines need more time for internalization of receptors

● TIMING

Steps 1 and 2, coupling of sulfo-SMCC to protamine: 3–4 h
Steps 3–7, desalting cetuximab before use: 1 h
Steps 8 and 9, coupling of cetuximab to sulfo-SMCC–protamine: 1 d
Step 10, desalting CSP: 1 h
Steps 11–13, measurement of protein content via Pierce BCA protein assay: 2 h
Steps 14–24, assessment of antibody functionality via flow cytometry (FACS analysis): 5 h
Step 25, coupling of CSP to siRNA: 3 h–1 d
Step 26, CSP siRNA payload assessment
Step 26A, gel shift assays: 4 h
Step 26B, fluorescence microscopy: 3 d
Step 26C, serum stability assay: 25 h
Step 27, downstream application of CSP–siRNA complexes
Step 27A, treatment of cells to obtain protein lysates for western blot analysis or other assays: 2–5 d
Step 27B, colony-forming assay (soft agar) to test for anchorage-independent tumor cell growth: 7–8 h (incubation time 1–2 weeks)
Step 27C, targeting and analyzing CRC cell lines in a xenograft model *in vivo*: 7–8 h (incubation time 4–8 weeks)
Box 1 (Steps 1–4), forming the GSP complex: 2 d
Box 1 (Steps 5–12), assessment of antibody functionality of the GSP complex via flow cytometry: 7–8 h
Box 1 (Steps 13–19), coupling of siRNA to GSP: 2–5 d

ANTICIPATED RESULTS

Here we describe a simple and efficient system for cellular targeting of EGFR-expressing tumor cells with siRNA by using an EGFR-specific mAb as a carrier molecule. As IgG does not bind and carry siRNA, we used a method of advanced chemical cross-linking to bind the IgG molecule to protamine, a cationic peptide. First, protamine is activated by an amino-terminal conjugation to sulfo-SMCC. As protamine is rich in arginine, there are ambient residues available. Next, this conjugate is coupled through cysteine residues to the IgG backbone (**Fig. 1**). As many of the more than 40 existing cysteines will be oxidized to form disulfide bridges that are important for IgG architecture or they will be inaccessible for steric reasons, only a minority of them will be decorated with sulfo-SMCC–protamine. The resulting CSP conjugate can be analyzed describing a number of physical and biochemical characteristics. Typically, it is a clear solution without any precipitations, and it can be stored at 4 °C for several weeks without loss of biological activity.

Binding of cetuximab causes the EGFR to internalize, and it induces a consequent block of EGF signaling followed by reduced viability and cell death in non-mutated tumor cells. This internalization process can be followed by flow cytometry. After the chemical coupling protocol shown in **Figures 1** and **2**, the conjugation of cetuximab with protamine does not interfere with the cetuximab binding and internalization of EGFR shown in **Figure 3b**. In FACS analysis with an antibody binding a different epitope on the large extracellular domain of EGFR, a clearance of EGFR from the cell surface can be observed regardless of whether cetuximab is modified by the chemical coupling procedure or not.

Protamine is conjugated to the IgG backbone to act as a binding and carrier molecule for siRNA driven by electrostatic interaction (**Fig. 1**). This ability is observed best by an electromobility shift assay: decreasing amounts of CSP were incubated with a constant amount of siRNA to depict a molar ratio where CSP is no longer able to bind more siRNA. This 'overflow' free siRNA can be visualized in an agarose gel, whereas the CSP–siRNA adduct lacks electromobility because of charge annihilation (**Fig. 4a**). Here, a value between the retarded 8 mol of siRNA per mol of CSP and the overloaded 20 mol siRNA per mol of CSP can be considered as the maximum siRNA load capacity for the given preparation of CSP.

The gain of apparent molecular weight of coupling protamine to cetuximab can be detected using SDS-PAGE. When it is applied to denaturing but nonreducing SDS-PAGE, CSP shows an apparent ~10% increased molecular weight compared with the non-modified IgG (**Fig. 3a**), suggesting a composition of 2–5 protamine molecules bound per mol of IgG. The complex is stable in serum for more than 24 h (**Fig. 4b**).

Western blotting

A hallmark of siRNA delivery is the ability of carrier molecules to release the siRNA after transmembrane transport to form the RNA-induced silencing complex necessary for efficient knockdown. Here we treated DLD-1 CRC cells *in vitro* with KRAS-specific esiRNA bound to CSP and subjected cell lysates to western blot analysis for KRAS. A substantial reduction of KRAS protein expression was detected in cells treated with KRAS siRNA, but not in cells with control esiRNA bound to CSP (**Fig. 5**).

Colony assays

An advantage of the antibody-based siRNA delivery is the ability to target cells growing in semisolid media—e.g., soft agar or methylcellulose for colony formation representing anchorage-independent growth. Here we repeatedly treated different CRC cell lines with CSP–siRNA (see Bäumer *et al.*[1]). With classic transfection, cells can only be treated once before seeding. In this case, a repeated treatment of cells is effective also during colony growth in semisolid medium.

Therapeutic application *in vivo*

Systemically applied CSP–siRNA was well tolerated in CD1 nude mice[1], and it led to substantial labeling of xenografted EGFR-positive CRC tumors with siRNA (**Fig. 6**). Treatment of cetuximab-resistant CRC tumor–bearing CD1 nude mice with KRAS-siRNA coupled to CSP led to marked tumor growth inhibition in comparison with control groups in KRAS-mutant tumors, but not BRAF-mutant tumors[1]. Growth inhibition was connected with reduced proliferation of treated tumors, as judged by Ki67 staining[1]. Our experiments indicate that this method of tumor cell–specific application of siRNA is totally dependent on EGFR expression and the oncogene equipment (i.e., active KRAS).

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AUTHOR CONTRIBUTIONS N.B., N.A. and S.B. wrote most of the protocol. N.B., N.A., L.T., F.B., C.R. and S.B. performed the experiments and analyzed the data. S.B., C.M.-T. and W.E.B. initially designed the protocol and supervised the study. All authors contributed to writing the paper.

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