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Hyper-dependence of breast cancer cell types on the nuclear transporter Importin β1

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ABSTRACT

We previously reported that overexpression of members of the Importin (Imp) superfamily of nuclear transporters results in increased nuclear trafficking through conventional transport pathways in tumour cells. Here we show for the first time that the extent of overexpression of Impβ1 correlates with disease state in the MCF10 human breast tumour progression system. Excitingly, we find that targeting Impβ1 activity through siRNA is >30 times more efficient in decreasing the viability of malignant ductal carcinoma cells compared to isogenic non-transformed counterparts, and is highly potent and tumour selective at subnanomolar concentrations. Tumour cell selectivity of the siRNA effects was unique to Impβ1 and not other Imps, with flow cytometric analysis showing >60% increased cell death compared to controls concomitant with reduced nuclear import efficiency as indicated by confocal microscopic analysis. This hypersensitivity of malignant cell types to Impβ1 knockdown raises the exciting possibility of anti-cancer therapies targeted at Impβ1.

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1. Introduction

Nuclear transport mediated by the Importin (Imp) superfamily of transport receptors is central to eukaryotic cell function, with regulated nuclear import and export of signalling molecules integral to processes such as transcription, translation, cell cycle progression and apoptosis. Imp expression is known to be altered in various types of cancers (such as cervical, breast, ovarian and lung) [1], but that this may impact on nuclear transport efficiency has only been demonstrated recently [2]. Importin β1 (Impβ1) is an important member of the Importin family of nuclear transporters that is highly expressed in transformed cells such as in Simian Virus 40 (SV40) large T-antigen-mediated transformation, Human Papillomavirus-16 (HPV-16) E6/E7-transformed epithelial cells and in gastric, bladder, breast and cervical cancer cell lines [2–5]. Highly conserved in mammals, and ubiquitously expressed in human tissues and cells, Impβ1 is critical for early embryonic development in the mouse, worm and fly [6,7] because of key roles in both interphase and mitosis [8]. Through its ability to mediate interaction of transport complexes with the nucleoporin (Nup) proteins of the nuclear pore complex (NPC), Impβ1 mediates nuclear import during interphase of a range of different proteins, including of cargoes bound directly to Impβ1 such as the signalling molecule parathyroid hormone-related protein PTHrP [9], cyclin B1 [10], chromatin remodelling factors such as SOX9 [11] and SRY [12] and basic loop helix factors such as activator protein-1 AP-1 and cAMP-response element-binding protein CREB [13]. Impβ1 can also mediate nuclear translocation of cargoes through the action of Impα adapters that directly recognise other cargoes, such as the tumour suppressor proteins pRb [14,15] and p53 [16], inducible transcription factors such as the signal transducers and activators of transcription (STATs) [17] and NF-κB family members [18]. Once in the nucleus, dissociation of the transport complexes is effected by binding to Impβ1 of the guanine binding protein Ran in activated GTP-bound form.

Impβ1 also plays a role after nuclear membrane breakdown during entry into mitosis, where either it alone or together with Impα regulates static spindle formation in a Ran-dependent manner by mediating the delivery of spindle assembly factors (such as the nuclear mitotic apparatus NuMA and microtubule associated TPX2 proteins) to spindle poles [19], and has indirect effects on dynamic microtubule attachment at kinetochores [20]. Impβ1 is critical to the formation of the NPC/nuclear envelope architecture during telophase through its ability to recruit Nups and other nuclear envelope components [21].

In this study we use an isogenic breast tumour progression model to show for the first time that overexpression of Impβ1 is a key contributor to increased Impα/β1-dependent nuclear transport activity, with a strong correlation between the extent of transport activity and tumour progression disease phenotype. Excitingly, we find that tumour cell types were highly sensitive to knockdown of Impβ1 but not various other Imps, implying that increased Impβ1 levels/activity may play a key role in tumour progression.

Abbreviations: Imp, Importin; Exp, Exportin; SV40, Simian Virus 40; NT, non-targeting; UT, untreated

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2. Materials and methods

2.1. Mammalian cell culture and siRNA treatment

The MCF10 breast tumour progression series was purchased from Prof. Fred Miller/Dr. Steven Santner at the Karmanos Cancer Institute, Detroit, MI, USA (under a materials transfer agreement which limits the use of the cell lines to the purchaser only). It is composed of the non-transformed MCF10A ductal breast epithelial cell line, its *Ha-Ras* ($G^{12}V$ mutated) transformed benign counterpart MCF10AT, and two fully malignant counterparts, the non-metastatic MCF10CA1h and metastatic MCF10CA1a cell lines generated by serial trocar implantation (passaging) in mice [22]. The 1BR3/1BR3.N human skin fibroblast cell pair [23] was obtained from Caroline Garrett (Centre for Genome Stability and Damage, Sussex, UK); the transformed derivative was generated by transfection with a plasmid containing SV40 genomic sequences for the early region composed of the transforming small and large T-antigen products.

MCF10A (passage < 75), MCF10AT (passage < 71), MCF10CA1h (passage < 60) and MCF10CA1a (passage < 105) cells were cultured in DMEM/F12 Ham's media supplemented with 5% horse serum (Invitrogen, Carlsbad, CA, USA), 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 0.5 μg/ml hydrocortisone, 10 μg/ml bovine insulin, 20 ng/ml human recombinant EGF and 100 ng/ml cholera toxin (all from Sigma-Aldrich, St. Louis, MO, USA), as previously, whilst 1BR3 and 1BR3.N cells (both at passages < 18) were cultured in DMEM supplemented with 15% FCS and 2 mM L-glutamine (Invitrogen), as previously [2].

For Impβ1 titration, comparative Imp silencing efficiency and cell killing assays in the MCF10 cell system, 2.3×10^5 cells were seeded into 6 cm dishes, treated 24 h later with ONTARGETplus SMARTPool siRNAs (pools of 4 different siRNAs from Dharmacon, GE Healthcare, Little Chalfont, UK) specific for Impα1, Impα3, Exp-1, CAS, Impβ1 or non-targeting control siRNA, where appropriate (see Supplementary Table S1 for siRNA sequences), at the indicated doses using RiboCellIn siRNA transfection reagent (BioCellChallenge, Toulon-Cedex, France) according to manufacturer's instructions, and incubated for 48 h (day 2) before re-seeding onto 96- (3.3×10^3 cells) or 6- (1.0×10^5 cells) well culture plates for XTT or confocal microscopic imaging and protein analysis respectively. Twenty-four hours later (day 3), cells were retreated with another dose of siRNA, and incubated for the indicated time points prior to Western, microscopic and/or XTT analysis.

2.2. Quantitative real-time reverse transcription-PCR

For analysis of mRNA expression levels, 0.7×10^6 cells were plated in 10 cm dishes and 48 h later RNA was isolated from cells using the RNeasy mini kit (Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's instructions, prior to DNAse digestion using RNAase-free DNAse (Ambion) and reverse-transcription by first strand cDNA synthesis using 500 ng of total RNA and Superscript III Reverse Transcriptase/random hexamers (Invitrogen). cDNA (20 ng) was then amplified using the SensiMix SYBR Master Mix (Bioline, Alexandria, NSW, Australia) and 1 μM Impβ1 forward and reverse primer mix (sense: 5′-AAGCCGCAGATTCTGTCAGT-3′; anti-sense: 5′-TTCCAAGCAG CTTTCCCTTA-3′). Quantitative RT-PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Life Technologies, Mulgrave, Victoria, Australia) for triplicate reactions. The comparative threshold cycle (C_T) method was used for the calculation of expression fold-change between normal and transformed cells, and normalised to GAPDH (sense: 5′-TGCACCACCAACTGCTTAGC-3′; anti-sense: 5′-GGCATGGACTGTGGTCATGAG-3′) and 18S (sense: 5′-TCCCCCAACTTCTTAGAGG-3′; anti-sense: 5′-CTTATGACCCGCACTTAC TG-3′) internal reference targets as determined from a set of tested genes by the *geNorm* function on qBase (not shown) [24].

2.3. Preparation of cell extracts and Western blotting

Cells were washed twice with ice-cold PBS prior to incubation in ice-cold RIPA lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) with fresh 5× EDTA-free complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA), scraped and lysed at 4 °C for 30 min and centrifuged at 10,000 *g* for 30 min at 4 °C to pellet insoluble material. The total protein concentration in each extract was estimated using the Bradford Dye Reagent (Bio-RAD, Hercules, CA, USA). Twenty micrograms of protein from each cell extract was then separated by SDS-PAGE (10% reducing gel), transferred onto nitrocellulose membrane (PALL Corporation, Port Washington, NY, USA) and probed with anti-Impα1, Impβ1, Exp-1, CAS monoclonal (all from BD Biosciences, San Jose, CA, USA) or anti-Impα3 polyclonal (Abcam, Cambridge, MA, USA) primary antibodies, followed by the appropriate host IgG-HRP secondary antibody (Chemicon, Temecula, CA, USA) according to the manufacturer's recommendations, and protein visualised using the Western Chemiluminescence Reagent (from Perkin-Elmer, Wellesley, MA, USA or Millipore, Bedford, MA, USA). The membranes were then stripped of antibody using Western strip buffer (25 mM glycine, 1% SDS, pH 2), blocked and re-probed with anti-α/β tubulin (Cell Signaling, Danvers, MA, USA) or -β actin (Abcam) antibodies followed by the appropriate host-IgG-HRP secondary antibodies and visualised as previously. The intensity of the resulting bands for all proteins was estimated by densitometry using an Alpha Imager (Alpha Innotec, Santa Clara, CA, USA) for image capture and the 1D electrophoresis gel analysis module from Image Quant TL software (GE Healthcare, Little Chalfont, Buckinghamshire, UK); results are expressed in terms of the ratio of the signal for Imp or Exp relative to that for α/β tubulin in transformed cells, relative to the respective value for the non-transformed cell line of the isogenic cell pair/set.

2.4. XTT assays

The effect of Impβ1 siRNA on cell proliferation was determined using the XTT assay as per the manufacturer's instructions. Briefly, siRNA-treated cell samples were re-seeded onto 96-well assay plates, retreated with siRNA (as per Section 2.1) and washed at the indicated time points, and the XTT/PMS reagents (Sigma-Aldrich) incubated in phenol-free DMEM/F12 Ham's media for 6 h before reading the change in absorbance at 690 and 450 nm using the FluoSTAR Optima plate reader (BMG LabTech). Specific absorbance was calculated by the following equation: (OD_{450} (sample) − OD_{450} (blank)) − (OD_{690} (sample) − OD_{690} (blank)) and given as the mean (±SD) absorbance calculated from 5 repeat wells/sample. The mean specific absorbance was normalised at each time point to that of the non-targeting siRNA-treated control in each cell line, and the value used as an indicator of cell viability.

2.5. DNA transfection, confocal microscopy and image analysis

The effect of Impβ1 siRNA on nuclear import efficiency was determined using confocal laser scanning microscopy of cells treated with siRNA as per Section 2.1 followed by transfection to express a βGal-GFP fusion protein with/without the cytomegalovirus polymerase pUL54 nuclear localisation sequence ($PAKKRAR^{1159}$) [25]. Cells were imaged live (×60 oil immersion objective, zoom of 1.0, 30 μm pinhole, 10% laser power, heated stage) 22–28 h pt using a Nikon TSI 100 confocal laser scanning microscope (Nikon, Tokyo, Japan). Image analysis was performed using the ImageJ v1.41 public domain software (U.S. National Institutes of Health, Bethesda, MD, USA) to determine the nuclear (Fn) cytoplasmic (Fc) and background (Fb) fluorescence. Briefly, a mean density measurement was made on a region of interest (ROI) of equal size (ROI = 30 arbitrary units) in the nuclear and cytoplasmic compartments, respectively, whilst the Fb measurements were made by placing the ROI on a non-transfected, autofluorescent region near

the cells' outer perimeter. The ratio of nuclear to cytoplasmic fluorescence (Fn/c) was then determined according to the formula: Fn/c = (Fn − Fb) / (Fc − Fb) [2].

2.6. Flow cytometric analysis and taxol treatment

For analysis of the effect of the siRNAs on cell death and the cell cycle, MCF10CA1h cells were plated at 2.3×10^5 cells in 6 cm dishes, treated with 10 nM Impβ1 or non-targeting siRNA (day 0) and 48 h later (day 2) detached and 6×10^5 cells seeded onto 10 cm dishes. The following day (day 3) cells were retreated with siRNA and fixed in 80% ethanol 48 and 72 h later (days 5 and 6 respectively) as required. In the case of the taxol-treated controls, cells were treated with 1 μg/ml taxol (Sigma-Aldrich) 3 h prior to fixation. Subsequent to fixing and RNase A treatment, cells were filtered and stained using propidium iodide (Sigma-Aldrich) to indicate the DNA content, and the cell cycle/death profiles (2×10^4 cells) were analysed using the LSR II flow cytometer (BD Biosciences). Quantification of the percentage of singlet cells at different stages of the cell cycle was performed using FlowJo software (TreeStar Inc., Ashland, OR, USA) and the Dean Jett Fox model.

2.7. Statistical significance

The significance ($p < 0.05$) of differences in results between transformed and non-transformed cells was determined using the Student's (or Welch corrected) *t*-test for unpaired data (2-tailed *p* value), as appropriate using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Hyper-dependence of transformed but not non-transformed cell types to Impβ1

Real-time RT-qPCR and Western analysis were applied to the MCF10 tumour progression model of invasive ductal carcinoma. The malignant MCF10CA1h and MCF10CA1a lines both showed significantly ($p < 0.05$) higher expression of Impβ1 at both the transcript (Fig. 1*a*) and protein (Fig. 1*b*) levels compared to their non-transformed counterpart, with the degree of overexpression correlating with the tumour progression state of the cells.

To test the effect of Impβ1 knockdown on cell proliferation and viability, we transiently treated the cells of the MCF10 series with 10 nM siRNA (pool of 4) specific to Impβ1 (Supp. Table S1), followed by detachment at 48 h post treatment (day 2), and retreatment with siRNAs 24 h later (day 3), prior to assessment of cell proliferation using the XTT assay (Fig. 2*a*) for up to 6 days post retreatment (days 3–9); direct cell counts (Supp. Fig. S1) yielded very similar results. Although treatment with Impβ1 siRNA decreased the viability of both malignant and non-transformed cells compared to untreated and non-targeting siRNA-treated controls (Fig. 2*a*), this effect was significantly ($p < 0.05$) more pronounced (c. 6-fold) in the transformed compared to the non-transformed cell types (Fig. 2*b*). Strikingly, low doses (0.5 nM) of siRNA failed to affect the viability of non-transformed cells, but decreased transformed/tumour cell viability by 50% relative to non-targeting siRNA-treated controls (Fig. 2*c*). Dose–response experiments as per Fig. 2*a* revealed that while nM doses of Impβ1 siRNA decreased the number of viable cells for both non-transformed and malignant lines, doses at, or below 1 nM resulted in selective effects on malignant but not non-transformed cells (Fig. 2*d*), with c. 33-fold higher siRNA potency in tumour compared to non-transformed cells (estimated absolute LD_{50} of 0.15 and 5 nM, respectively). To confirm that the effects were attributable to decreased Impβ1 function, we employed a different approach, using the Impβ1-specific inhibitor 2,4-diaminoquinazoline Importazole (Ipz), which inhibits Impβ1's role in nuclear import [26]. Significantly, Ipz effectively decreased the viability of malignant MCF10CA1h much more than that of non-transformed MCF10A cells, in a similar manner to siRNA (Supp. Fig. S2), clearly implying the specificity of the effects observed for the Impβ1-specific siRNA approaches.

Treating cells with increasing concentrations of Impβ1 siRNA (Fig. 3*a*) revealed that Impβ1 silencing was c. 20-fold more efficient in malignant MCF10CA1h cells than non-transformed MCF10A cells at day 5 (log growth-phase; Fig. 3*b*, absolute IC_{50} of 0.066 compared to 1.3 nM, respectively), with greater silencing efficiency also seen in benign and malignant MCF10AT and MCF10CA1a lines respectively (Fig. 3*cd*). The increased efficiency of Impβ1 silencing in the transformed lines was not attributable to increased siRNA uptake since all lines took up siRNA to the same extent (Supp. Fig. S3).

To confirm the findings in the MCF10 cell system, we used an additional isogenic transformed cell system; the normal primary human skin fibroblast cell line 1BR3 together with its SV40 large T-antigen-transformed counterpart 1BR3.N [23]. Cells were treated with 1 and 10 nM Impβ1 siRNAs and viability measured at day 6 using the XTT assay. Consistent with the findings above, 1 nM Impβ1 siRNA decreased viability of the transformed 1BR3.N, but not primary 1BR3 cells (Supp. Fig. S4*a*); Western/densitometric analysis confirmed efficient silencing of Impβ1 expression in both primary and transformed cells (Supp. Fig. S4*bc*).

Overall, the results suggest that low doses of Impβ1 siRNA selectively decrease viability of different transformed and malignant cell types but not primary/non-transformed counterparts.

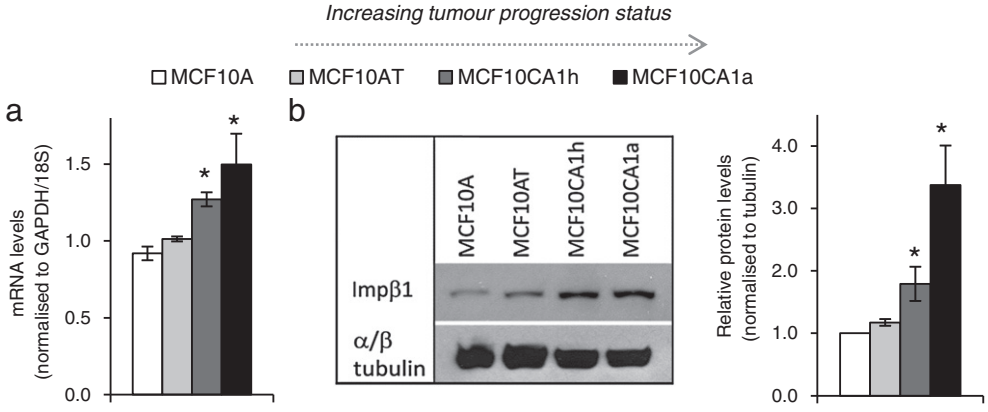

Fig. 1. Elevated expression of Impβ1 in malignant breast cancer cells. (a) Levels for Impβ1 mRNA in the MCF10 tumour progression series as detected by real-time qRT-PCR. Results represent the mean ± SE ($n = 4$), for expression normalised to the geometric mean of GAPDH and 18S internal reference targets in transformed (grey or black bars) and non-transformed cells (white bar). * $p < 0.05$ *vs.* MCF10A cells. (b) Western (left) and densitometric (right) analysis of Impβ1 levels in the MCF10 cell series. Results represent the mean ± SE ($n = 6$) for the ratio of the intensity of the signal obtained for Impβ1 normalised to that for α/β tubulin control protein in transformed, relative to non-transformed cells. * $p < 0.05$ *vs.* MCF10A cells.

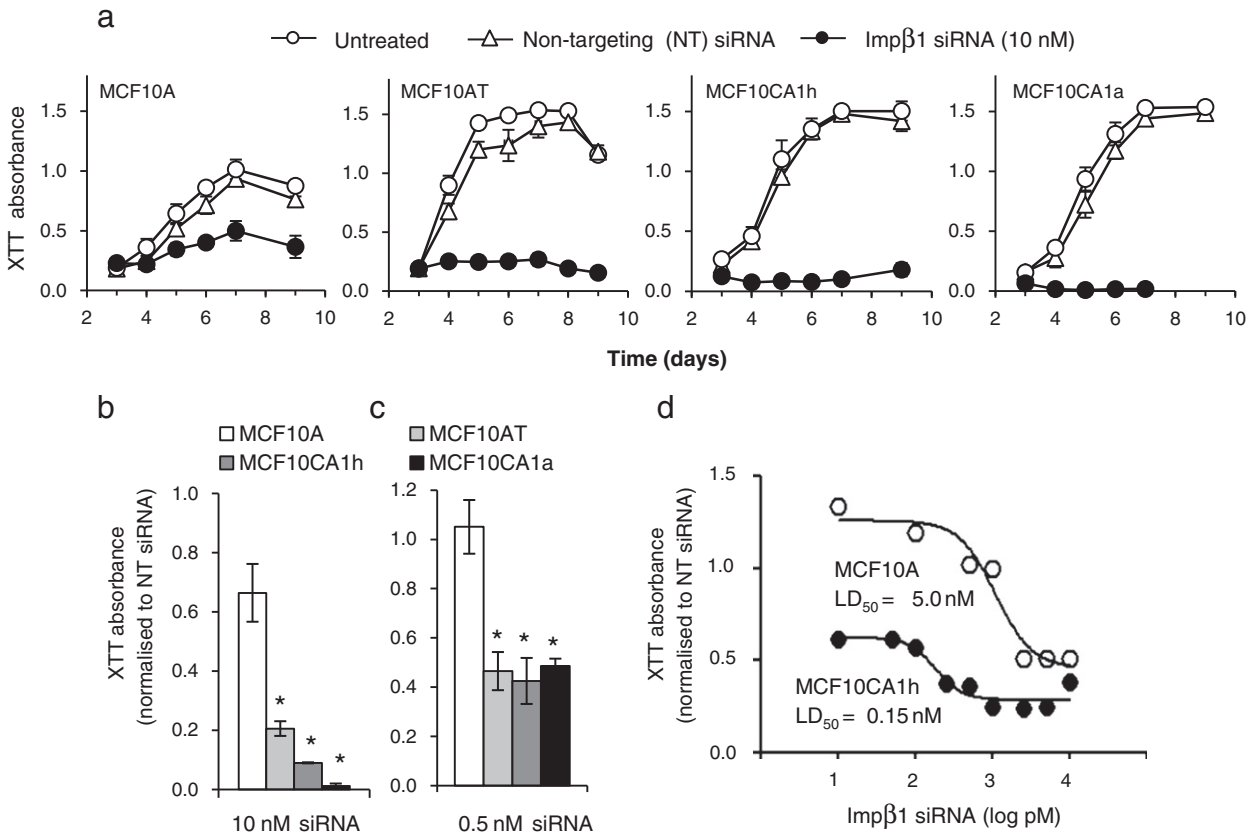

Fig. 2. Transformed but not non-transformed breast cell types are hyper-dependent on Impβ1. (a) XTT assays for cells of the MCF10 cell series treated twice with 10 nM Impβ1 or non-targeting (NT) siRNA, or for untreated (UT) controls at the indicated time points after replating. Results for XTT absorbance are for the mean ± SD from a single representative experiment in a series of three experiments. (b) Results for cell viability measured on day 5 from (a) in non-transformed (white bars) and malignant cells (grey/black bars) and (c) in cells treated with 0.5 nM siRNA. Results represent the ratio of the mean ± SD absorbance relative to NT siRNA-treated cells. * $p < 0.0001$ *vs.* MCF10A cells. (d) LD_{50} curves on day 5 for non-transformed (white spheres) and malignant (black spheres) cells treated twice with the indicated concentrations of Impβ1 siRNA. Results are from a single typical experiment in a set of two independent experiments.

3.2. Tumour-selective targeting activity is specific to Impβ1 siRNA

To test whether the tumour selectivity of Impβ1 siRNA induced killing activity reflected a general hypersensitivity of tumour cells to silencing/inhibition of components of the cellular nuclear transport machinery, we treated non-transformed MCF10A and malignant MCF10CA1h cells twice with siRNAs (pools of 4 different siRNAs) targeting Imps other than Impβ1 (Supp. Table S1), including Impα1, α3, β1, CAS (Cse1l) or Exp-1 (Crm-1) at 1 or 10 nM as required to induce efficient gene silencing, and monitored viability (Fig. 4*a*). Although silencing of Exp-1 and to some extent CAS resulted in significantly decreased viability ($p < 0.05$) compared to non-targeting siRNA-treated controls, none of the Imp/Exp siRNAs other than that for Impβ1 ($p < 0.05$) affected the malignant cells selectively (Fig. 4*a*). Impβ1 was efficiently silenced in malignant (c. 50%) but not in MCF10A cells (0%) as expected (1 nM siRNA), whilst all of the other Imp and Exp proteins showed similar silencing efficiency across both cell types (Fig. 4*bc*). Based on these data, malignant cells appear to be uniquely hypersensitive to knockdown of Impβ1, and not affected specifically by siRNA to other Imps/Exps.

3.3. Impβ1 siRNA induces death in malignant cells

Given that the transformed and malignant MCF10 cell types exhibit c. 2-fold shorter doubling times than the non-transformed MCF10A line (Supp. Fig. S5) and are hypersensitive to Impβ1 knockdown (see Section 3.1), and that Impβ1 is known to regulate multiple aspects of mitosis [20,27], we investigated whether the decreased viability in Impβ1 siRNA-treated cells may be associated with a block in cell cycle/mitotic progression and whether this relates to the induction of cell death. To this end, we performed propidium iodide staining of malignant MCF10CA1h cells treated twice with 10 nM Impβ1 siRNA followed by flow cytometry (Fig. 5*a*) and DNA content analysis (Fig. 5*b*) on day 4 or day 5 corresponding to time points of the XTT assay in Fig. 2. Impβ1 siRNA significantly ($p < 0.05$) increased the sub-G1 population compared to untreated and non-targeting siRNA-treated controls on day 5 but not day 4 (Fig. 5*b*). No significant differences were observed in the G2/M or S phase populations in cells treated with Impβ1 or non-targeting (control) siRNA on either day (Fig. 5*b*). In contrast, cells that had been treated with the microtubule-stabilising agent taxol showed an increase and decrease in the G2/M and G1 populations respectively, indicative of G2/M phase arrest (Fig. 5*b*). These data suggest Impβ1 siRNA's growth inhibitory effects on malignant cells are due to the induction of cell death, but do not relate to perturbation of cell cycle progression.

3.4. Impβ1 siRNA decreases nuclear import efficiency in malignant but not in non-transformed cells

Impβ1 is known to mediate nuclear protein import in interphase cells [19]. We visualised the effects of Impβ1 siRNA (10 and 1 nM) on nuclear accumulation of a large, c. 480 kDa βGal-GFP-fusion protein (βGal-NLS-GFP) containing the Impα/β1-recognised NLS from the human cytomegalovirus DNA polymerase catalytic subunit pUL54 [25] or βGal-GFP alone in non-transformed MCF10A and malignant MCF10CA1h cells treated twice with Impβ1 or non-targeting control siRNA on day 6, by live cell confocal laser scanning microscopy (Fig. 6*a*), and used image analysis to determine the nuclear-to-cytoplasmic-

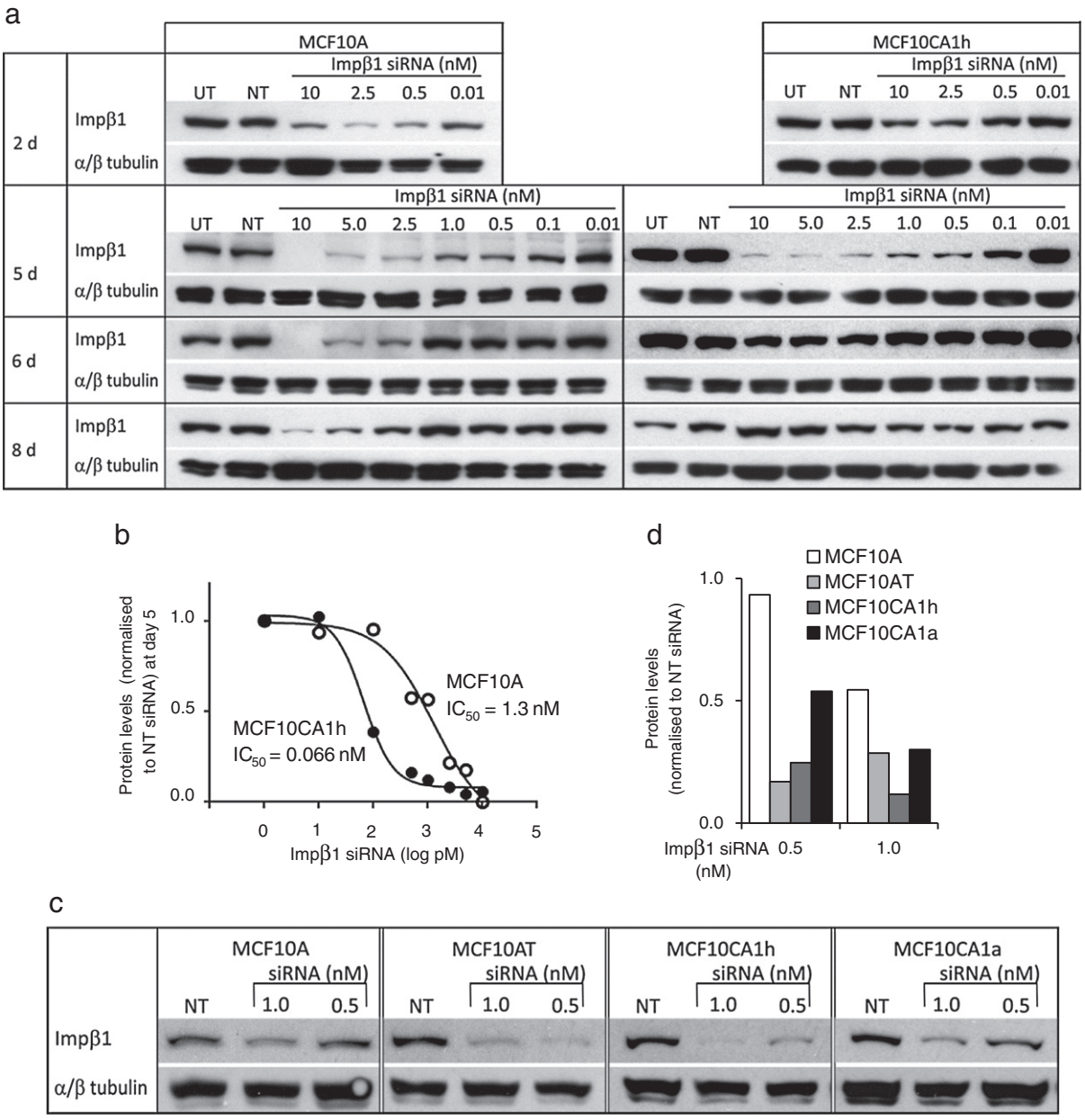

Fig. 3. Increased silencing of Impβ1 in transformed breast cells compared to non-transformed cells. Analysis of Impβ1 expression in the MCF10 series treated with Impβ1 siRNA. (a) Results for Western analysis for Impβ1 expression in non-transformed MCF10A (left panel) and malignant MCF10CA1h (right panel) cells treated twice with increasing concentrations of Impβ1 siRNA or a non-targeting (NT) siRNA, and untreated (UT) controls at the indicated assay time points (compare to Fig. 2a for corresponding XTT time course values). Results are from a single typical experiment in a set of two independent experiments. (b) Densitometric analysis from images such as those shown in (a). Results represent the mean for the level of protein expression in Impβ1 siRNA-treated samples, normalised to NT siRNA-treated controls at day 5 in non-transformed (white spheres) and malignant (black spheres) cells. The IC_{50} is given for both cell types, log pM = 0 represents the value for the NT-condition. (c) Western and (d) densitometric analysis for effect of 0.5 or 1.0 nM Impβ1 siRNA on Impβ1 levels in the MCF10 cell series. Results are from a single typical experiment in a set of two independent experiments.

fluorescence ratio (Fn/c; Fig. 6*b*). The level of βGal-NLS-GFP nuclear accumulation was significantly ($p < 0.0001$) c. 5-fold decreased by Impβ1 siRNA-treatment in malignant MCF10CA1h cells compared to non-targeting siRNA treated controls, bringing the extent of nuclear accumulation to levels identical to those in the non-transformed MCF10A cells (Fig. 6*ab*); Impβ1 siRNA did not impact on nuclear accumulation in the latter. Consistent with the idea that the impact of Impβ1 knockdown on βGal-NLS-GFP transport was not due to effects at the level of the nuclear envelope/NPC, the βGal-GFP control protein remained strongly cytoplasmic in the presence or absence of Impβ1 siRNA treatment in both cell lines (Fig. 6*ab*). The clear implication is that the higher levels of Impβ1 in the malignant cells are directly responsible for the higher nuclear accumulation efficiency observed, as compared to non-transformed cells.

4. Discussion

We show here for the first time that Impβ1 siRNA is highly potent in inhibiting the proliferation of basal type tumour cells arising from ductal epithelia and transformed primary cells, but not their non-transformed isogenic counterparts, although at higher (≥10 nM) doses we observed partial decreases in viability even in normal/non-transformed cell types. Intriguingly, this hypersensitivity is evident even though malignant cells express higher levels of Impβ1 transcript/protein [2–5]. That the results are not unique to siRNA approaches is indicated by the hypersensitivity of malignant cells to a small molecule inhibitor of Impβ1 (Supp. Fig. S2).

Impβ1 plays a central role in nuclear trafficking of important signalling molecules such as transcription factors through action alone, or as a

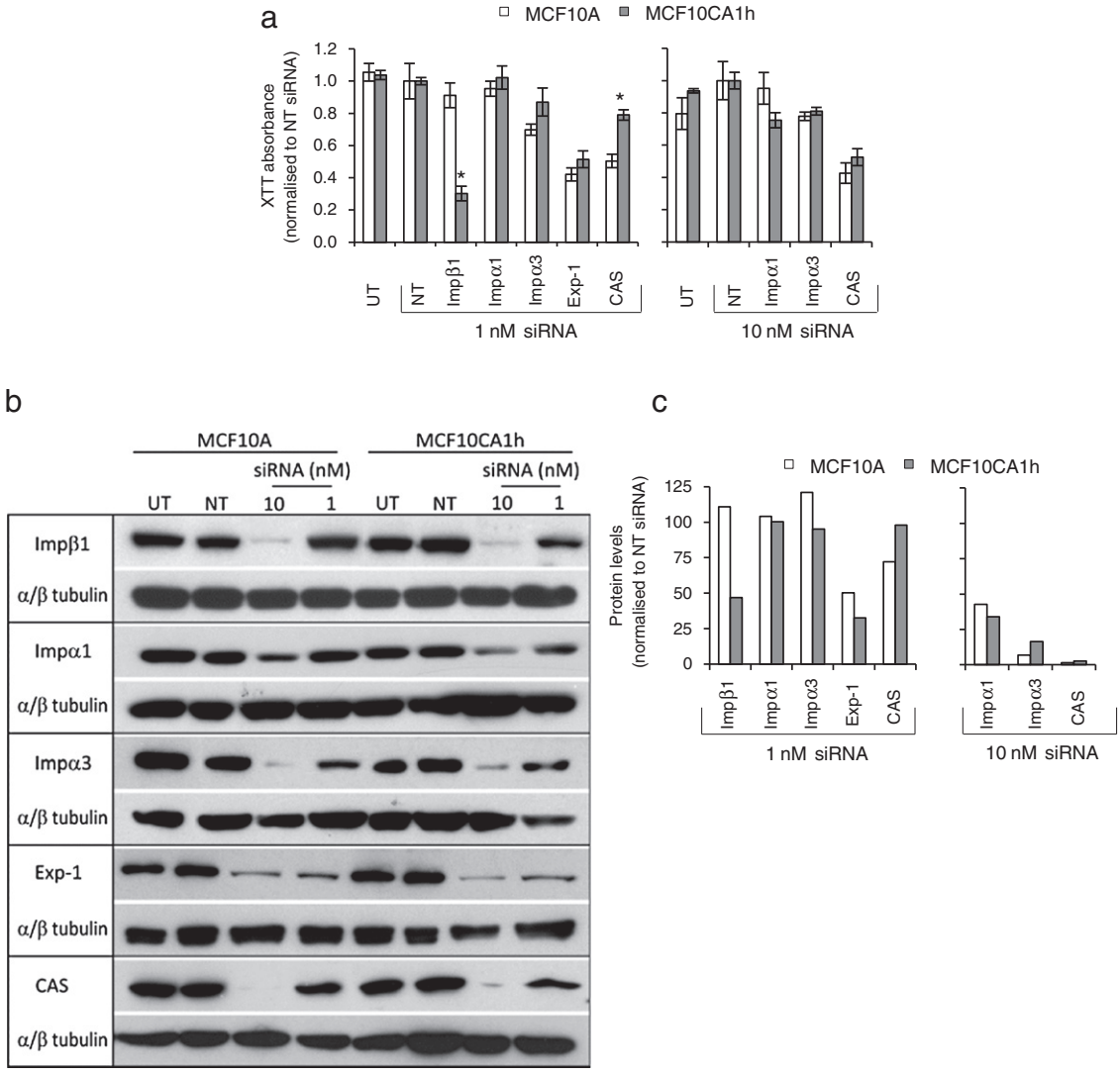

Fig. 4. Tumour-enhanced silencing and cell killing activity is specific to Impβ1 siRNA. (a) Viability of non-transformed MCF10A and malignant MCF10CA1h cells treated twice with 1 (left) or 10 (right) nM siRNA for the indicated Imp/Exp or non-targeting (NT) control was measured using the XTT assay on day 5 as per Fig. 2b. * $p < 0.05$ *vs.* MCF10A cells. (b) Western and (c) densitometric analysis of Imp expression in samples from (a) in cells treated with 1 or 10 nM siRNAs as per Fig. 3. Results are from a single representative experiment in a series of ≥ 4 separate experiments. UT, untreated.

heterodimer with adaptors such as Impα, snurportin [28] and even Imp7. It is also a key driver of mitotic exit and G1 phase progression. We found that treatment of cells with Impβ1 siRNA selectively decreased nuclear import efficiency and induced cell death in malignant but not in non-transformed cells, without significant effects on the number of cells undergoing mitosis. The precise mechanism of tumour cell hypersensitivity to reduced levels of Impβ1 is not clear, but presumably relates to these key cellular roles of Impβ1 distinct from those of other Imps/Exps, and the fact that faster growing tumour cells, although expressing higher levels of Impβ1, are more dependent on ("addicted to") Impβ1 than non-transformed cells. Comparable tumour cell "addiction" has been observed for other cellular factors, oncogenic or otherwise, such as cyclin D1 [29], c-myc [30], DNA damage response kinases ATM [31] and CHK1 [32], and the reactive oxygen species (ROS)-inhibiting pyruvate dehydrogenase kinase [33], whereby targeting these factors by RNAi or small molecular inhibitors results in selective cancer cell death. As for these factors, increased Impβ1 levels may be critical to support oncogenic tumour promoting functions driving cell cycle progression and proliferation in transformed cells, whilst being less critical in non-transformed cell types. The results here suggest that the mechanistic basis is the reliance on elevated Impα/β1-dependent nuclear import efficiency by malignant cells; that Imp levels can contribute critically to tumour promotion has been shown for the oncoprotein c-myc and the transcription factor E2F1 [34,35].

The basis of the increased sensitivity of the tumour cells to Impβ1 knockdown at low siRNA concentrations is not clear, especially as the breast tumour cell types in this study replicate faster than non-transformed MCF10A cells, which can result in the dilution of siRNA, and have a higher abundance of Impβ1 transcript and protein in untreated cells, both of which can negatively impact RNAi efficiency [36,37]. Significantly, this is clearly not a generalised mechanism, as we found that silencing of other Imp and Exp proteins was similar in MCF10CA1h tumour compared to non-transformed cells thus improved silencing may relate to inherent differences in target recognition/individual siRNA potencies etc.

Targeting the components of the nuclear transport system through inhibition of nuclear transporter activity is already underway in preclinical and clinical trials for SINEs (selective inhibitors of nuclear export), which are small molecular inhibitors that target the activity of the Impβ1-homologue Exp-1 (Crm-1) [38], although the realistic utility of these novel leptomycin B [39] like molecules in a clinical context remains to be established, predominantly of their high toxicity [40]. Our studies in transformed breast and skin cells indicate that targeting Impβ1 using siRNA is highly toxic to tumour but not non-transformed

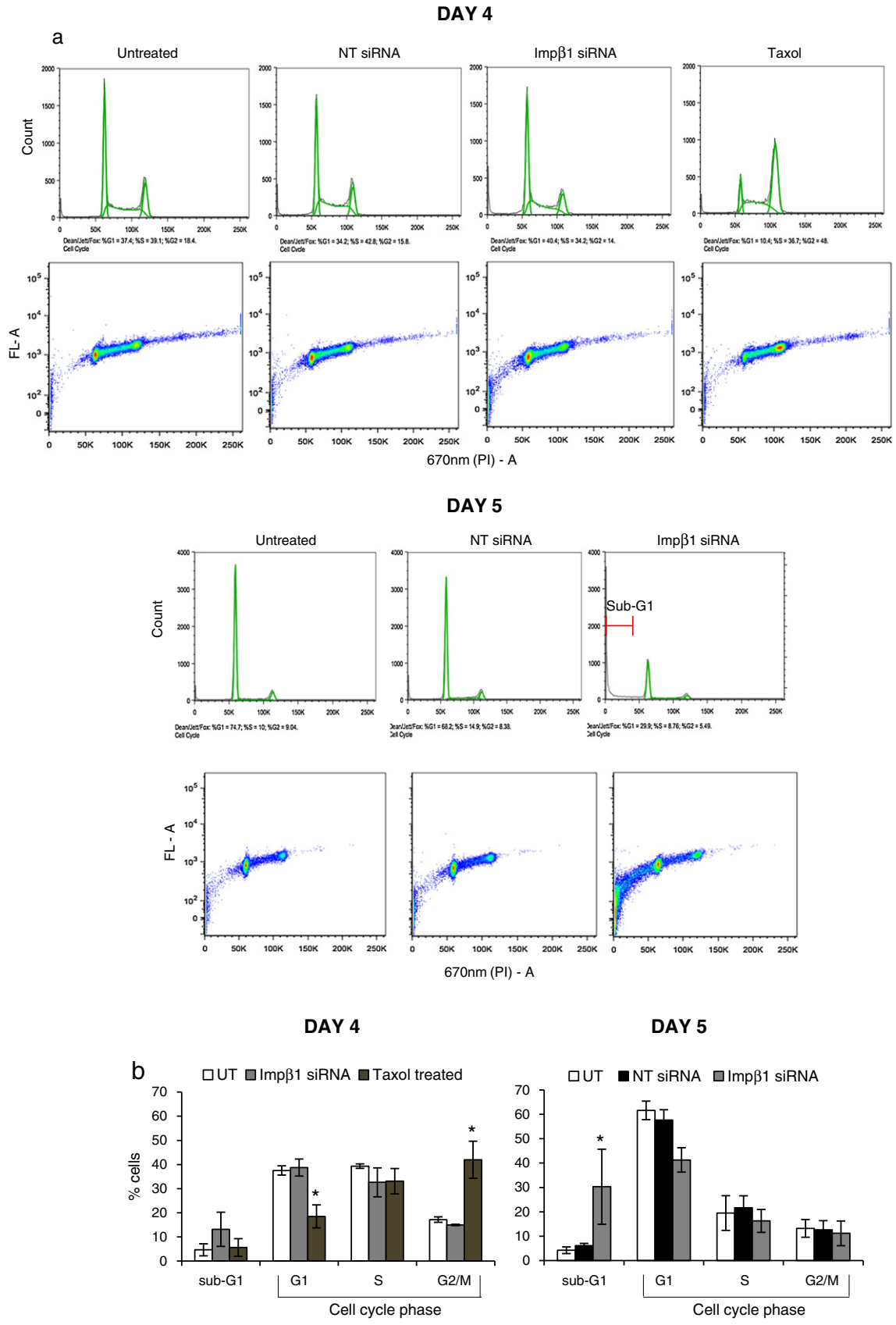

Fig. 5. Knockdown of Impβ1 induces cell death. (a) Flow cytometric analysis of the DNA content and cell cycle in malignant MCF10CA1h cells treated twice with 10 nM Impβ1 or non-targeting (NT) siRNAs at day 4 or 5 corresponding to the XTT assay in Fig. 2 as indicated, from a single representative assay in a series of three separate experiments. (b) Cell cycle and sub-G1 population analysis of samples from (a) at day 4 or 5. Results are given as % of cells in sub-G1/the indicated cell cycle phases, representing the mean ± SE from a series of three experiments. * $p < 0.05$ *vs.* NT siRNA-treated cells.

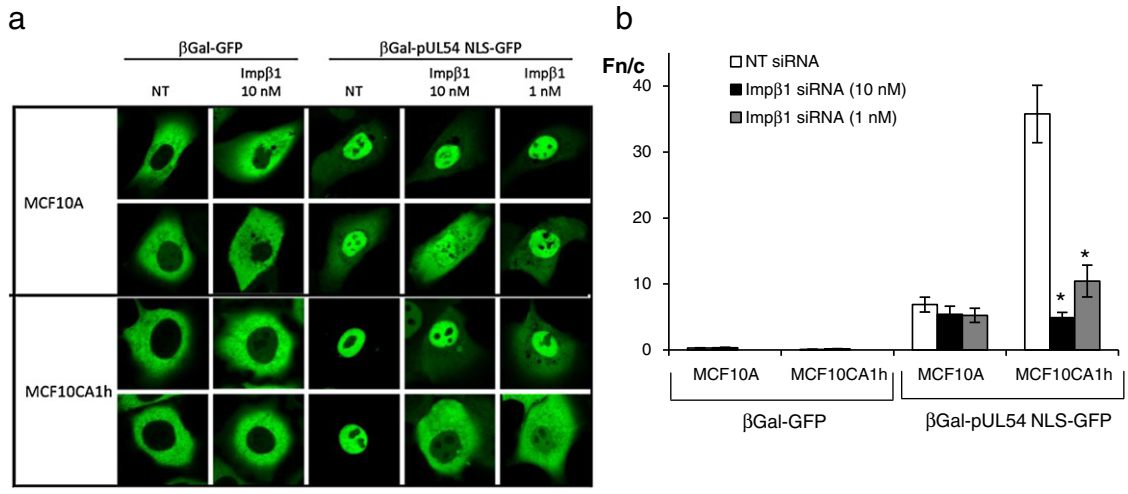

Fig. 6. Impβ1 knockdown decreases nuclear import efficiency in malignant but not non-transformed cells. (a) Malignant MCF10CA1h and non-transformed MCF10A cells were treated twice with 10 or 1 nM siRNAs for Impβ1 or non-targeting (NT) controls and were transfected at day 5 to express an Impα/β1-recognised NLS-containing fusion protein (βGal-pUL54 NLS-GFP) or βGal-GFP alone, and imaged live 24 h post transfection by confocal laser scanning microscopy (CLSM). (b) Image analysis of images such as those shown in (a) was used to determine the nuclear-to-cytoplasmic fluorescence (Fn/c) ratio. Results are for the mean ± SE ($n > 55$), from a single typical experiment in a series of three experiments. * $p < 0.05$ *vs.* NT siRNA-treated cells.

cell types, which raises the question of whether Impβ1 siRNAs may prove useful in other cancer cell and animal models, and whether targeting of Impβ1 may also be used in therapy. That the anti-proliferative activity of tumour suppressors such as p53, pRb, BRCA1 and $p27^{kip}$ is strongly dependent on nuclear localisation through Impα/β1 [14,16,41,42] is a potential concern in this context, but since many of these suppressors are inactivated in highly malignant cell types due to genetic abnormalities [43], this is presumably not a major factor. Indeed, the present study indicates the striking hypersensitivity of malignant cells to approaches targeting Impβ1, consistent with other published observations for lung carcinoma, head and neck carcinoma cells [44] and cervical cancer lines [4,45]. Whether targeting Impβ1 transport activity by small molecular inhibitors or siRNAs can induce selective tumour killing *in vivo*, is an exciting question for future consideration.

5. Conclusion

In conclusion, this is the first comprehensive demonstration of hypersensitivity of isogenic tumour compared to normal cell types to Impβ1 inhibition in a disease progression model of human basal type breast carcinoma. Future work in this laboratory is aimed at pursuing the observations here to assess their potential application in tumour selective therapies.

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Transparency Document

The Transparency document associated with this article can be found, in the online version.

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