Differential role of D cyclins in the regulation of cell cycle by influencing Ki67 expression in HaCaT cells

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Abbreviations: BPE, bovine pituitary extract; FBS, fetal bovine serum; MTT, Thiazolyl Blue Tetrazolium Bromide; siRNA; small interfering RNA

Abstract

D-type cyclins are important regulatory proteins of the G1/S phase of the cell cycle however, their specific functions are only partially understood. We show that silencing of individual D-type cyclins has no effect on the proliferation and morphology of Immortalized non-tumorigenic human epidermal (HaCaT) cells, while double and triple D cyclin silencing results in the failure of the cytokinesis leading to the appearance of large multinucleated cells. Both CDC20 and Ki67 mRNA is downregulated in these cells. Ki67 mRNA silenced cells show similar multinucleated cellular phenotype as double or triple D cyclin silenced cells without affecting D cyclin expression, suggesting that Ki67 is necessary for normal G2/M transition. Our data have revealed that cyclin D1 may have a leading role in G1/S phase regulation and suggest an incomplete functional overlap among D cyclins. Our results indicate that beside their well-known functions during the G0-G1/S phase, D-type cyclins play a pivotal role in the regulation of mitosis via influencing Ki67 expression in a downstream manner probably through E2F1 activation in HaCaT cells.

INTRODUCTION

Cell cycle is one of the most controlled biological process where cyclins play a key role in the regulation. In mammals, the family of D-type cyclins has three members named D1, D2 and D3 [1, 2]. Although these proteins are encoded by separate genes on different chromosomes, they show high amino acid similarities, suggesting a common ancestor gene [3, 4]. D-type cyclin regulatory function is linked to Cyclin-dependent kinase 4 (Cdk4) and Cdk6 complex formation in G0/G1 phase [5-7] and phosphorylation of the retinoblastoma protein (pRB) leading to its inactivation, triggering the transcription factor E2F1-dependent gene induction resulting in cyclin E/Cdk2 kinase activation [7]. G1 interval can be shortened by enforced overexpression of D-type cyclins in *in vitro* cultured fibroblasts [8, 9]. Silencing of D cyclins or blocking their function with antibodies interferes with G1 phase progression in certain cells, without affecting other cell cycle intervals [9, 10]. It has been shown that cyclin D1 also influences G2/M transition. On the one hand, in the G2 phase high level of cyclin D1 is achieved by Ras activity, which remains elevated during the M phase [11, 12]. On the other hand, cyclin D1 depletion during G2/M phase activates the Checkpoint kinase 1 (Chk1)-Cdc2 DNA damage pathway as shown in *in vitro* experiments under oxidative stress [13].

Data suggest that beside their cell cycle regulatory functions, D-type cyclins have further functions. Cyclin D1 plays a role in cellular metabolism, fat cell differentiation and cellular migration [14] and has additional functions in cellular invasion, angiogenesis, DNA binding, regulation of transcription factors, DNA damage recognition and repair, initiation of chromosomal destabilization and feedback governing expression of the noncoding genome [15]. Cyclin D2 regulates macrophage activation [16, 17] and spermatogonial

differentiation [17], while cyclin D3 has a role in the terminal differentiation of cells respectively [18].

Knockout experiments in murine models suggest that the functions of different D cyclins are not entirely redundant. While „Cyclin D1 only" mice develop megaloblastic anaemia, „cyclin D2 only" mice show neurological abnormalities, and „cyclin D3 only" mice have abnormal cerebella[1, 2]. Cyclin D1 deficient mice display hypoplastic retinas, underdeveloped mammary glands and present developmental neurological abnormalities. At the same time knock-in mice expressing cyclin D2 in place of D1 show nearly normal development of retina and mammary glands suggesting that cyclin D2 can partially replace the function of cyclin D1 in the retina [19] . Both cyclin D1/Cdk4 and cyclin D3/Cdk6 have a common substrate, the hub protein, nucleolin. The lack of nucleolin leads to the accumulation of cells in the G2 phase, centrosome control deficiencies, nuclear abnormalities (multiple nuclei, micronuclei, large nuclei) and apoptosis in HeLa cells and in human primary fibroblasts. CyclinD1/Cdk4, is associated with centrosome amplification (CA) and is a key regulator of centrosome duplication. Recently it has been revealed that Ki67 is essential for nucleolin- mitotic chromosome association [20] and chromosome architecture development [21, 22]. Knockdown of cyclin D1 significantly elevates cells accumulating in the G1 phase thereby inhibiting cell proliferation [23]. Silencing of cyclin D3 prolonged G2 phase and mitosis in HeLa cells. Compromised chromosome migration to the metaphase plate results in mitotic exit with an increased number of HeLa cells containing multiple or micronuclei, indicating an important function for cyclin D3 activity in the S/G2 phase [24]. In bronchial epithelial (BEAS) cells the knockdown of each D-type cyclin caused cell culture growth delay. Triple silencing of D-type cyclins resulted in growth suppression indicating that functional interactions are needed for efficient cell growth in BEAS cells [25] .

Previously we have shown that the regulatory functions of D-type cyclins in keratinocytes may not be entirely redundant. D1-type cyclin expression is the highest during the G0-G1/S phase, while cyclin D2 and D3 dominates during the repeated rapid turnover of the highly proliferating HaCaT cells[26]. The differential expression of these cyclins suggested that they may have specific functions beside regulating the cell cycle in these cells [26]. To get further insight into the function of D-type cyclins in HaCaT cells we have used multiple gene-specific silencing. As long as two D type cyclins are functional, cells exhibit unaltered morphology, proliferation and mitosis, indicating that D cyclins can at least in part compensate for the lack of each other in cell cycle regulatory functions. In contrast, after double or triple silencing of D-type cyclins the amount of cells in S phase was elevated and the formation of multinucleated cells was observed. At the same time a massive decrease in CDC20 and Ki67 expression was detected in double or triple D cyclin silenced cells. Moreover, Ki67 silencing alone resulted in similar mitotic abnormalities without affecting mRNA expressions of D-type cyclins.

We also used a modified mathematical model of G1/S transition combined with a model for D cyclin silencing. The original model simulates G1/S transition, which is prolonged with the decrease of D cyclin mRNA which is well in line with our in vitro data. The mathematical model shows that G1/S transition results in low phospho-retinoblastoma (pRb) and high E2F1 levels. At sufficient inhibition of Cyclin D transcription - and thus translation - when a threshold of D cyclin protein level is reached the G1/S transition is prohibited with constantly high level pRb and low level E2F1. Lack of sufficient E2F1 may cause Ki67 deficiency, since it is known that E2F1 has two binding sites on the promoter of Ki67 [27]. Our in vitro experiments combined with mathematical modelling could explain the formation of multinucleated cells when either Ki67 or D cyclins are insufficiently available.

MATERIALS AND METHODS

Cell culture

The spontaneously immortalized human keratinocyte cell line (HaCaT), kindly provided by Dr. N. E. Fusenig (Heidelberg, Germany) [28], was cultured in 75 cm2 cell culture flasks and maintained in high glucose Dulbecco's modified Eagle's medium (high glucose DMEM; Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; HyClone, Perbio, Budapest, Hungary), L-glutamine, and antibiotic/antimycotic solution containing 100 U/ml penicillin, 100 µl/ml streptomycin and amphotericin B (all from Sigma-Aldrich, Steinheim, Germany) at 37°C in a humidified atmosphere containing 5% CO2. For setup of the Ki67 overexpression model the cells were divided into two groups. HaCaT cells were contact inhibited for 1 day and serum starved for further 5 days (Group2). In Group1 cells were released from contact inhibition and seeded under 70% confluency in FBS containing medium. Both cell groups were nucleofected with D1D2D3 cyclin siRNAs in suspensions and then plated and cultured. The mRNA expression of Ki67 was detected 24 hours after nucleofection.

Gene silencing by siRNA

After forming a monolayer we performed a serum starvation for 5 days on HaCaT cell cultures. Cells were then harvested and nucleofected in suspension with siRNAs using NucleofectorTM II (Amaxa Biosystems, Koeln, Germany) and V Kit optimized for HaCaT cells (VCA 1003, Amaxa Biosystems). Cells were then plated onto 12 well tissue culture plates and cultured in antibiotic free medium containing 10% FBS. Cyclin D1 siRNA (sc 29286), Cyclin D3 siRNA (sc 35136), Ki67 siRNA (sc 37613), siControl RNA (sc 36869) all from Santa Cruz Biotechnology, INC (Santa Cruz, CA, USA) and CCND 2 (Cyclin D2)

SMARTpool siRNA (M 003211) and CCND2 siControl (D-001206-13) from Dharmacon Permoscientific Upstate (CO, USA) were used following the instructions of the manufacturers. Following cell plating samples were taken at 24, 48, 72, 96 and 168 hours for analysis. Transfection efficiency was determined by flow cytometry using a plasmid encoding the enhanced green fluorescent protein eGFP (Amaxa Biosystems). Treated cells of each group were counted on 3 randomly chosen fields of images taken with Nikon Eclipse TS-100 light microscope equipped with Nikon Coolpix 4500 digital Camera. The total number of cells and the number of multinucleated cells were counted in each experiment and data are presented as percentage +/- standard deviation (s.d.) of multinucleated cells.

Real-time RT-PCR

Total RNA was isolated from HaCaT cells using TRIzol^{TM} reagent (Gibco, Invitrogen, Carlsbad, CA, USA,) according to the manufacturer's instruction. First strand cDNA was synthetized from 1µg of total RNA using the iScriptTM cDNA Synthesis Kit (Bio Rad, Hercules, CA). Real-time RT-PCR experiments were performed to quantify the relative abundance of each mRNA by using the iCycler IQ Real-Time PCR machine (BioRad). Primers specific for cyclin D1 (Cat. Hs 00277039), D2 (Cat. Hs 00153380) and D3 (Cat. Hs 00236949) were provided by Applied Biosystems (Foster City, CA, USA). Primers specific for Ki67 were the following: forward: CAA GAC TCG GTC CCT GAA AA, reverse: TTG CTG TTC TGC CTC AGT CTT (Nucleic Acid Synthesis Laboratory, Biological Research Centre, Hungarian Academy of Sciences). The abundance of each gene of interest was normalized to the expression of 18S ribosomal RNA. The primers for 18S were the following: forward: CGG CTA CCA CAT CCA AGG AA, reverse: GCT GGA ATT ACC

GCG GCT, TaqMan probe: TexRed TGC TGG CAC CAG ACT TGC CCT C BHQ 1 (Integrated DNA Technologies, Szeged, Hungary).

Human Cell Cycle PCR Array

A microarray based transcriptional profiling was used to compare the cyclin D1, D2 and D3 silenced cells to nucleofected controls. The Human Cell Cycle RT2 Profiler™ PCR Array (SA Biosciences, Budapest, Hungary) was used following the instructions of the manufacturer. Hierarchical clustering based on the gene expression levels was performed by R software [29].

Immunocytochemistry

HaCaT cells were synchronized and nucleofected as described above. Cells were seeded onto 8 chamber polystyrene vessel tissue culture treated glass slides (Falcon CultureSlide, Becton Dickinson Labware, Sigma-Aldrich, Steinheim, Germany). Cultured cells on slides were fixed in 2% paraformaldehyde for 20 minutes and were rinsed with Tris buffered saline (TBS, Sigma) for 5 minutes. Non-specific staining of cells was blocked by pre incubation with 0.5% bovine serum albumin diluted in TBST (TBS, 0.1% Triton X, Reanal, Budapest, Hungary) for 30 min at room temperature (RT). Cells were then incubated overnight (ON) at 4°C with the primary antibodies. Purified monoclonal antibodies to cyclin D1 (clone SP4, RM-910), cyclin D3 (clone DCS-22, MS-215-R7) all from NeoMarkers, Fremont, CA and cyclin D2 (10R-C149a) from Fitzgerald Industries Int. Inc. Concord, MA, USA were applied at 1:200, 1:25 and 1:1 dilutions respectively, rabbit IgG (NeoMarkers), mouse IgG1 and mouse IgG2a (Sigma) were used as isotype controls. Slides were then incubated with fluorescent conjugated secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG for cyclin D1 and Alexa Fluor 647 goat anti-mouse IgG for cyclin D2 and D3, all

at 2.5 μg/ml final concentration) for 3 hours at RT, followed by incubation with 4, 6-diamidino-2-phenylindole-dihydrochloride (DAPI, Sigma, at 1 μg/ml final concentration) for 15 minutes. For Ki67 staining, slides containing cytopreparations were fixed in 99.97% aceton for 5 minutes. Non-specific antigen blocking was performed in 1% BSA-TBS for 30 minutes at RT. EGFR (clone EP38Y, rabbit, LabVision Corporation, Fremont, CA, USA) and Ki67 (clone B56, mouse, Histopathology Ltd., Pécs, Hungary) primary antibodies were applied at 1:100 and 1:200 dilutions for 30 minutes and 15 minutes at RT, respectively. After washing in TBS for 3x5 minutes, secondary goat anti-rabbit IgG Alexa 488-conjugated (Life Technologies/Molecular Probes, Invitrogen) and goat anti-mouse IgG Alexa 546-conjugated (Life Technologies/Molecular Probes, Invitrogen) were used at a dilution of 1:500 for 3 hours at RT. After washing in TBS for 3x5 minutes, DAPI nuclear staining was applied at a concentration of 1 μg/ml for 20 minutes at RT. Finally, slides were washed and coverslipped in Fluoromount mounting medium. Immunostaining was visualized using a Zeiss Axio Imager microscope.

Flow cytometric analysis of HaCaT cells

HaCaT cell suspensions were prepared using 0.025% Trypsin (Sigma) in phospate-buffered saline (PBS). Cells were fixed in 70% ethanol at -20°C ON. The following monoclonal antibodies were used: purified mouse anti-Ki67 (clone B56, Histopathology Ltd.) in 1:100 dilution, mouse IgG at the same concentration served as isotype control (Sigma). As secondary antibody anti-mouse IgG coupled with fluorescein isothiocyanate was used in 1:200 dilutions (BD Pharmingen, San Diego, CA, USA). Cells were washed in PBS containing 0.5% BSA (Sigma) and 0.1% Triton-X 100 (Reanal) and incubated with the primary antibody at 4°C ON. After being washed in PBS containing 0.1% Triton-X 100, cells were incubated for 30 minutes at RT with the secondary antibody and analysed by

FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and CellQuest software (Becton Dickinson). Results were expressed as the geometric mean fluorescence intensity (GeoMean) calculated by subtraction (GeoMean of the antigen-specific antibodies stained cells minus GeoMean of isotype-matched control antibodies stained cells) and normalized to control siRNA treated cells.

MTT (Thiazolyl Blue Tetrazolium Bromide) assay

HaCaT keratinocytes were contact inhibited for 1 day and serum starved for 5 days. Cells were then trypsinized and nucleofected in cell suspension with cyclin D1, D2 and D3 siRNAs. HaCaT keratinocytes were seeded into wells of 96 well plates directly after transfection at a density of 5 x 10^3 cells/well and grown in 200 µl complete high glucose DMEM medium for 72 hours. For the MTT assays, the medium was replaced with 0.5 mg/ml Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma) in RPMI (Sigma) without phenol red. The cultures were incubated for 4hours at 37°C. Living cells degrade MTT by mitochondrial succinate dehydrogenase, resulting in formation of MTT formazan. The converted dye was solubilized with acidic isopropyl alcohol (0.04 M HCl in isopropyl alcohol, Reanal). The optical density of the wells was determined using a microplate reader (Multiscan EX, ThermoLabsystems, San Diego, USA) at 540 nm. Values were normalized to the mock siRNA nucleofected controls.

APO2.7 – PE staining

HaCaT keratinocytes were contact inhibited for 1 day and serum starved for 5 days. Cells were then harvested and nucleofected with cyclin D1, D2 and D3 siRNA in suspensions and then plated into 12 well tissue culture plates and cultured in antibiotic free medium containing 10% fetal bovine serum. Cells were harvested by trypsinization and washed

twice in PBS. Cell density was adjusted to $1x10^6$ cells/ml, then 1 ml cell suspension was centrifuged at 200 g for 6 minutes at RT. The pellet was resuspended in 100 µl cold 4°C 100 µg/ml digitonin in PBSF (PBS with 2.5% FBS), and incubated for 20 minutes on ice. Two ml of cold (4°C) PBSF was added and cells were centrifuged at 200 g for 6 minutes at RT. The pellet was resuspended in 20 µl of Apo2.7 PE (Coulter, Immunotech, Marseille, France) and 80 µl of PBSF and incubated for 15 minutes in the dark at RT. After this, 2 ml of PBSF was added and cells were centrifuged at 200 g for 6 min at RT. The pellet was resuspended in 1 ml of PBSF. Analysis was carried out using a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson).

Propidium iodide DNA staining

HaCaT keratinocytes were contact inhibited for 1 day and serum starved for 5 days. Cells were then harvested and nucleofected with cyclin D1, D2 and D3 siRNAs in suspensions and then plated into 12 well tissue culture plates and cultured in antibiotic free medium containing 10% fetal bovine serum. HaCaT cells were harvested by trypsinization and washed twice in PBS. Cell density was adjusted to $1x10^6$cells/ml then 1 ml cell suspension was centrifuged at 1500 rpm for 9 minutes. The pellet was resuspended in 70% cold (20°C) ethanol and fixed for at least 24h at 20°C. After fixation, cells were centrifuged at 3200 rpm for 9 minutes and washed in PBS. Cells were then resuspended in 500 µl PI/RNase staining buffer (containing 50 µg/ml PI and 100 U/ml RNase A from BD) and left for 30 minutes at RT. The samples were analysed using a FacsCalibur machine (Becton Dickinson) and evaluated by Modfit software (Verity Software House, Topsham, ME, USA).

Co-staining HaCaT cells for Ki67 and Propidium iodide

HaCaT cells were synchronized as described below. 0h, 24h, 48h and 72 hours after releasing cells we collected samples for Flow analysis. HaCaT cell suspensions were prepared using 0.025% Trypsin (Sigma Aldrich, Saint Louis, Missouri, USA) in phospate-buffered saline (PBS). Cells were partitioned into FACS tubes ($2x10^5$ cells/FACS tube) washed in PBS and were centrifuged at 3200 rpm for 10 minutes, at 4°C. For fixation, Fixation/Permeabilization working solution (eBioscience, Vienna, Austria; 200 ul/tube) were used for 25 minutes, at 4°C, than were permeabilized in 1x Permeabilization Buffer (eBioscience, Vienna, Austria; 500 ul/tube) for 5 minutes, at 4°C. After fixation and permeabilization, cells were washed in PBS, and were centrifuged as above described. The pellet was resuspended in PBS containing 0,5% BSA (Sigma Aldrich, Saint Louis, Missouri, USA) and the primary antibody: purified mouse monoclonal anti-human Ki67 (clone:B56, BD Pharmingen, San Diego, CA, USA, 100 ul/tube, in 1:250 dilution) or mouse IgG1 at the same concentration served as isotype control (BioLegend, San Diego, California, USA) and were incubated for 45 minutes, at room temperature. The cells were washed in PBS and were centrifuged as above described. The pellet was resuspended in PBS containing 0,5% BSA (Sigma Aldrich, Saint Louis, Missouri, USA) and the secondary antibody: goat anti-mouse Alexa Flour 647 (Invitrogen, Carlsbad, California, USA, 100 ul/tube, in 1:500 dilution) for 45 minutes, at room temperature, than the cells were washed in PBS, and were centrifuged as above described. After this immunostaining, the pellet was resuspended in a mix in PBS (300 ul/tube) containing Propidium-iodide (Becton Dickinson, Franklin Lakes, New Jersey, USA; 0,2 ug/$2x10^5$ cells), and RNase A (Sigma Aldrich, Saint Louis, Missouri, USA; 0,1 ug/$2x10^5$ cells) and left for 30 minutes at RT, than were washed in PBS, were centrifuged as above described, and were resuspended in PBS (400 ul/tube). The samples were analysed using a BD FACSAria™ Fusion machine (Becton Dickinson, Franklin Lakes, New Jersey, USA).

High content imaging system and image analysis

Olympus IX83 microscope with Olympus ScanR screening platform was used for image acquisition and fully automated analysis of all images taken from all experiments. The assay setup was based on cytometry orientated gating and classification populations of cells. First cells with completely bounded nuclei stained by DAPI were gated as first ROI, based on the fluorescent intensity of staining and circularity of the objects as a threshold. According the DAPI staining the ScanR built in Cell Cycle Assay was used to determine G1, M, G2 phases of cell cycle and 2n, 4n content of cells. Following the 2n and 4n gating, Ki67 positive cells were distinguished and quantified. A reference gallery of objects of the 4n selected population was made, randomly selected pictures were further analysed by ImageJ for a three-dimensional graph of the intensities of pixels to visualize the slight differences (**Figure 5.**).

Bioinformatical analysis

Simulation of G1/S transition was done in MATLAB with Simbiology Toolbox (Release 2012b, The MathWorks, Inc., Natick, Massachusetts, United States). Model was downloaded from Biomodels [30].

RESULTS

Single gene specific silencing of cyclin D2 and D3 in HaCaT keratinocytes

To examine whether D2 and D3 cyclins perform unique roles in the proliferation of HaCaT cells, their gene expressions were specifically silenced. D2 (**Fig.1A**) and D3 (**Fig.1B**) siRNAs significantly downregulated the expression of cyclin D2 and D3 mRNA in the transfected HaCaT cells leaving the mRNA expression of other D-type cyclins unaffected. Cell numbers determined by MTT assay were similar in all cultures. Cell and colony morphology in D2 and D3 cyclin-silenced HaCaT cultures did not show any obvious difference compared to the control cells (data not shown).

Multiple silencing of D-type cyclins in HaCaT keratinocytes

Next we addressed the question whether cells in which two or three D type cyclins were silenced at the same time were able to keep their proliferative and morphological characteristics. Double silencing of D-type cyclins in all three combinations (D1D2, D1D3 and D2D3) resulted not only in the massive reduction in mRNA levels of the silenced cyclins (**Fig.1C**) but also in the disappearance of the specific proteins based on immunostaining (**Fig.1D**), while no alteration was detectable for the remaining non-silenced D cyclin. Following triple silencing (D1D2D3) mRNA of all three D cyclins were heavily decreased and none of them were detectable by immunostaining (**Fig.1D**).

72 hours after nucleofection and release from cell quiescence large, multinucleated cells were apparent in all four multiple D cyclin silenced cultures (**Fig.2A**). After 96 hours growing number of small vacuoles in the cytoplasm of these aberrant cells was observed (**Fig.2A**). We counted the number of multinucleated cells in the different cultures 72 hours after the nucleofection (n=5). Cells with aberrant characteristic features were present in

2.4+/-0.25% in siD1D2, 2.25+/-0.22% in siD1D3, 1.8+/-0.01% in siD2D3 and 7.56+/-0.37% in siD1D2D3 treated cultures. In controls aberrant cells were: 0.16+/-0.04% in siControl for siD1D2 and siD2D3, 0.11+/-0.07% in siControl for siD1D3 and 0.1+/-0.05% in siControl for siD1D2D3. Triple silencing of D cyclins resulted in more than 3 times of multinucleated cells than any of the double silencing (**Fig.2B**). Double silencing of D-type cyclins caused growth suppressions by 35% in cyclin D1 and D2, 35.8% in cyclin D1 and D3 and 22% in cyclin D2 and D3 silenced cells, while repression of all three D-type cyclins cooperatively suppressed HaCaT cell growth by 38% compared to the mock siRNA nucleofected controls in the 72-hour samples (**Fig.2C**). To determine the effect of multiple D-type cyclin silencing on cell cycle progression, propidium iodide DNA analysis was performed. Seventy-two hours after nucleofection - at the time of the appearance of large multinucleated cells - we measured an increased number of cells in S phase in the double and triple D cyclin silenced cells (**Fig.2D**): 48.94% of cyclin D1-D2-D3, 50.48% of cyclin D1-D3, 44.24% of cyclin D2-D3-silenced and 39.35% of control siRNA treated cells were in S phase. At later time points (at 96 and 168 hours), the same tendency was detectable: at 96 hour 45.67% and at 168 hour 26.06% of the triple D cyclin-silenced cells were in S phase compared to 40.81% at 96 hour and 15.91% at 168 hour of the control siRNA treated cells (data not shown). Flow cytometric analysis revealed no apoptotic effect on these cells as measured by Apo2.7 staining **(Suppl. Fig 1A)**.

D-type cyclins regulate the expression of Ki67 in HaCaT keratinocytes

To determine genes influenced by double and triple silencing of D-type cyclins during cell cycle, we applied SuperArray PCR. Twenty-four hours after nucleofection the expression of 84 genes were examined including cyclins and their associated Cdks, Cdk inhibitors, Cdk phosphatases, and cell cycle checkpoint molecules involved in the control of cell division.

Hierarchical clustering of genes based on their gene expression level clearly separated the Cyclin D1D2D3 triple and cyclin D1D3 double silenced cells from their controls and the other double or single silenced cells which were also separated according the combinations, but formed a higher cluster with their controls (**Fig.3**). In this clustering one of the most consistent and significant decrease was found in the mRNA expressions of Ki67 and CDC20 in the double and triple silenced cells compared to siControl treated cells which finding was validated by RT-PCR respectively (**Fig.4A and Suppl. Fig. 1B**). In siD2D3 cells the mRNA expression of Ki67 was higher compared to other double or triple D cyclin silenced cultures (**Fig.4A**). Moreover, Ki67 protein was not detectable by immunostaining in the large multinucleated cells in siD1D2D3 cultures, while a strong Ki67 protein expression was apparent in both the untreated control and control siRNA treated cultures (**Fig.4B**). Ki67 was suggested to be expressed mainly in the G2/M phase of the cell cycle and it is often used as a proliferation marker [31], until now the effect on chromosomal development is described [21, 22], the other exact functions of Ki67 on the cell cycle regulation is not well detailed. To better understand its function we investigated the effect of Ki67 silencing on mitosis in HaCaT cells. 72 hours post nucleotransfection silencing of Ki67 resulted in the appearance of multinucleated aberrant cells with similar characteristics to those found after double and triple silencing of D cyclins (**Fig.4C**). In siKi67 treated cultures 5.76+/-0.37% of cells showed the aberrant characteristic features mentioned above (n=5) in comparison to 0.1+/-0.09% cells found in siRNA controls. Ki67 siRNA significantly downregulated the expression of Ki67 but it had no effect on the mRNA expressions of cyclin D1, D2 and D3 24 hours after the nucleofection (**Fig.4D**).

To differentiate whether the lack of Ki67 was a consequence of reduced cell cycle entry or whether it was a target of Cyclin D we quantified the DAPI staining and indicated which cells are 4n G2 cells - and their Ki67 levels by an automated high content imaging screening

system (**Fig.5A and C**). The number of Ki67 positive cells within the 4n cell population was decreased upon D1D2D3 cyclin silencing compared to siRNA control resulting in a 33.5% decrease (**Fig.5B**).

Nearly quiescent non-dividing (100% confluent) serum starved HaCaT cells showed a massive reduction in the protein expression of Ki67 as indicated by flow cytometric measurements. These cells were then released from contact inhibition and seeded under 70% confluency in the presence of serum. This highly proliferative state was associated with high level of Ki67 expression (**Fig.6A**). Flow cytometric analysis also showed that Ki67 expression gradually increased in G2M phase cells (1.85±0.49% at 0h, 2.30±0.28% at 24h, 21.70±8.63% at 48h and 64.00±3.82% at 72 hours after releasing cells (**Suppl. Fig. 2A, B**).Using this model system of low and high Ki67 conditions we examined the effect of D1D2D3 cyclin triple silencing on the cellular phenotype. Cells were divided into two groups. High Ki67 expression was achieved by growing cells to 70% confluency in FBS containing medium (group 1), whereas cells grown to 100% confluency and serum starved showed low Ki67 expression determined by Real-time RT-PCR (group 2) (**Fig.6B**, 0h). Cells were nucleofected with D1D2D3 cyclin siRNAs in suspensions and then plated and cultured. In group 1, the mRNA expression of Ki67 was similar to siControl treated cells 24 hours after nucleofection (**Fig.6B**), while in group 2 Ki67 mRNA remained at low levels. 72 hours after the nucleofection the number of the appearing large multinucleated cells relevantly decreased in the Ki67 overexpressed - D1D2D3 cyclin silenced cell cultures compared to triple D cyclin silenced synchronized cells: in group 1, the rate of large multinucleated cells was only 1.5+/-0.29%, while in group 2 this rate was 7.12+/-0.49%.

The presence of multinucleated cells cannot explain decreased cell count in cyclin D silenced samples

We addressed the question whether decreased cell count at 72 hours in Cyclin D silenced cultures was due to the presence of non-dividing multinuclear cells or is caused also by other factors like elongated cell cycle or exit from cell cycle. To test this, we used a mathematical approach.

If the actual cell count at 72 hours is x, then it is $(1-d)x$ in siRNA-transfected cells, where d is the proportion of decrement of transfected cell count compared to control. The number of multinucleated cells is $m(1-d)x$, where m is the proportion of multinuclear cells in transfected samples. If cell duplication time in control samples is 24 hours, then there are $\frac{x}{2^3}$ cells at 0 time point, and there are $\frac{(1-d)(1-m)x}{2^3}$ cells with normal morphology in Cyclin D silenced samples. As equal cell count were plated for each sample:

$$(1)\ \frac{x}{2^3} = \frac{(1-d)(1-m)x}{2^3} + (1-d)xm,$$

if only the presence of multinuclear cells explain the decreased cell count in cyclin D silenced samples. We know that there is no cytokinesis of multinuclear cells so their number at 0 time point is the same as at 72 hours. If

$$(2)\ \frac{x}{2^3} \neq \frac{(1-d)(1-m)x}{2^3} + (1-d)xm,$$

then cell turnover of cells with normal morphology is different from control.

After solving the formula for the silenced samples, we found that the cell count in all samples is lower, which could be explained only by the presence of non-dividing multinuclear cells (Table 1). One possibility is that the elongated cell cycle explains our findings. If we hypothesize that this is the main factor, which is responsible for the decreased cell count in silenced samples, we can approximate the mean length of cell cycle using the previous formulas. We have to solve the following equation:

$$(4)\ \frac{x}{2^3} = \frac{(1-d)(1-m)x}{2^n} + (1-d)xm,$$

where n equals the number of cell duplications in the normal cells of Cyclin D silenced sample. The value of n can be calculated with the following formula:

$$(5)\ n = \log_2 \frac{(1-d)(1-m)}{0.125 - (1-d)m}$$

If normal cell cycle is 24 hours long then cell duplication time is:

$$(6)\ \frac{3}{n}24 = \frac{72}{n}$$

We approximated cell cycle length and found the highest elongation in D1D3 silenced samples (**Table 1.**).

Decrement of cell number (d), fraction of multinuclear cells (m) at 72 hours post-transfection in Cyclin D silenced samples compared to control. The calculated left side and right side values for equation 2 are shown. If we assume, that cell cycle elongation is mainly responsible for lower cell count in silenced samples, we can approximate the length of cell cycle using equation 4, 5 and 6. n shows the average number of cell duplications and 72/n is the average length of cell cycle.

Elongated cell cycle is a probable explanation of lower cell count in silenced samples

In order to examine the effect of siRNA load on cell cycle, we applied the mathematical model of G1/S transition developed by Swat et al.[31] and integrated the inhibitory effect of siRNA on Cyclin D synthesis into the model (**S2 Dataset**). Using this model we calculated the effect of different siRNA concentrations on the G1/S transition. Parallel with increasing siRNA concentrations G1/S transition delay increased, reflecting decreased pRb and enhanced E2F1 levels. At threshold the G1/S transition did not occur, pRb level remained high and E2F1 level remained low (**Fig.6C.**). The simulation results suggest that the cell cycle is elongated in D cyclin silenced cells with normal morphology. Additionally, D

cyclin siRNA concentrations reach a threshold in some cells resulting in aberrant cytokinesis and the formation of multinuclear cells. According to Swat's mathematical model this effect is probably caused by low E2F1 level. These findings suggest elongated cell cycle as a probable explanation of lower cell count in silenced samples. However, we cannot exclude the partial role of other factors like exit from cell cycle.

DISCUSSION

D-type cyclins were demonstrated to play a role in the G1 phase of cell cycle by regulating the function of the retinoblastoma susceptibility gene product (Rb) [7, 32, 33]. Several reports showed that the expression pattern of D-type cyclins varies in different cell types during cell cycle [18, 34]. Although there is some degree of functional redundancy among D-type cyclins in certain cell types, it is becoming evident, that all D-type cyclins have special functions as well [15, 35-37]. It has been demonstrated that the expression of D1 cyclin is not restricted to the G1 phase although its level drops at the S phase, it shows an increase in the G2 phase which peaks at the M/G1 phase in actively proliferating cells [12, 38, 39]. We have previously shown that D-type cyclins are differentially expressed in synchronized HaCaT cells in certain phases of the cell cycle. D1-type cyclin expression is the highest during the G0-G1/S phase, while cyclin D2 and D3 dominates during the repeated rapid turnover of the highly proliferating HaCaT cells [26].

In our previous experiments we have also demonstrated that knocking down the expression of cyclin D1 had no effect on the proliferation and morphology of HaCaT keratinocytes during the interval between cellular quiescence and intense proliferation after release from quiescence suggesting that D2 and D3 cyclins could substitute for D1 in driving keratinocyte cell cycle [26.]. In this paper we demonstrated that similar to D1 cyclin silencing knocking down cyclin D2 and D3 alone has no effect on the proliferation and morphology of these cells. Silencing of individual D cyclins did not result in a quantitative compensatory increase of the other two D-type cyclins. Similar observation was made in mice, where cyclin D2 and D3 can compensate for each other in driving the cell cycle of B lymphocytes [40, 41]. Furthermore, cyclin D2 could compensate the loss of cyclin D1 in knock out experiments, where estrogen-induced mouse uterine epithelial cell proliferation was investigated [42]. In Cyclin D3 knockout animals the skeletal muscle stem cells showed

increased proliferation, differentiation and reduced self-renewal capability[43]. *In vitro* single silencing of D-type cyclins with small interfering RNAs resulted in cell growth suppression in human bronchial epithelial (HBE) cells respectively [25]. In adipogenesis the silencing of cyclin D1, D2, or D3 blocked the differentiation, and influenced the mitotic clonal expansion of 3T3-L1 cells [44].

The fact that knocking down individual D cyclins had no effect in HaCaT cell proliferation and morphology in our experiments may be explained by the fact that these cells are immortalized keratinocytes. Alternatively, the requirement for individual D-type cyclin functions in cell cycle regulation may vary among different cell types. In mammalian embryonic fibroblasts normal cell cycle can take place even without the cyclin D-Cdk4/6 complex [43]: in these cells cyclin E-Cdk2 alone is able to phosphorylate and inactivate pRb, activate E2F, and induce DNA synthesis [45].

Contrary to the single knockdown experiments, double and triple silencing of D-type cyclins in HaCaT cells resulted in the formation of large multinucleated cells, higher number of cells in S phase and moderate growth suppression as determined by cell counts. Similar to the single cyclin silencing, no compensatory increase of the remaining D-type cyclins was detected in these cells. Interestingly, growth suppression and the formation of multinucleated cells was observed in all double and triple silenced cultures however, when cyclin D1 was present the cell proliferation and morphology was less affected, indicating that among D cyclins, cyclin D1 plays a leading role in cell cycle regulation. Our data suggest that the lack of two or three D cyclins could contribute to a higher number of cells in the S phase. In HBE cells, dual knockdown of D-type cyclins resulted in growth suppression, however double or triple silencing of D-type cyclins had no effect on cell morphology [25]. Similar to our observation in HaCaT cells, no compensatory overexpression of the remaining D-type cyclins was observed in HBE cells [14].

UV-irradiation triggered inhibition of cyclin D3 results in G2 phase arrest in HeLa and A2058 cells indicating that cyclin D3 has an important regulatory function for cell cycle progression through G2 phase into mitosis [36]. Small interfering RNA knockdown of cyclin D3 delayed the progression in HeLa cells through G2 phase, these cells failed to undergo cytokinesis correctly and showed an aberrant mitotic phenotype characterized by lagging chromosomes that formed micromultinucleated cells [24].

Here we show that double or triple knockdown of D cyclins also resulted in a decreased CDC20 mRNA expression. CDC20 is a known oncoprotein that is part of the Anaphase Promoting Complex (APC). It is known that CDC20 silencing results in G2/M phase arrest leading to suppression of cancer cell growth [46].

Furthermore, the lack of double or triple D cyclins led to Ki67 downregulation but when cyclin D1 was present the level of Ki67 was the least affected and the number of multinucleated cells was the lowest, further supporting a superior role of cyclin D1 and suggesting an incomplete functional overlap among D cyclins in cell cycle regulation. Although Ki67 is an established extensively used proliferation marker, its precise function is not well described. Ki67 is known to accumulate in cells from the G1-phase until cells exit mitosis after which its level decreases and becomes nearly undetectable [47]. Ki67 is a phosphatase 1-interacting protein that plays a role in the regulation of the nucleolar protein B23/nucleophosmin [20]. Phosphorilation of Ki67 during mitosis is associated with the condensation of the chromosomes and separation of sister chromatids [21, 22][39]. Ki67 is essential for the formation of normal perichromosomal layer[21, 22], the lack of Ki67 leads to abnormally small nuclei in HeLa cells [20]. In MCF7 cells, Ki67 shows a partial overlap in localization with nucleolin and fibrillarin [48]. Nucleolin has multiple functions: it plays a major role in the ribosome biogenesis being a major component of the nucleolus and it also has important non-nucleolar functions. Nucleolin is a known substrate for cyclin D-Cdk

complexes. The lack of nucleolin leads to cell growth arrest, accumulation in the G2 phase, abnormal nuclei (micronuclei and defected control of centrosome duplication) [49]. Well in line with our observation, it has been shown that knockdown of Ki67 by small interfering RNA leads to inhibition of proliferation in human renal carcinoma cells [50]. Here we have found that although Ki67 silencing did not affect the expression of D type cyclins, it resulted in the appearance of aberrant cells with very similar morphology to double and triple D cyclin silenced cells. The formation of multinucleated cells in Ki67 silenced HaCaT keratinocytes suggests that Ki67 has a cell cycle regulatory function during the mitosis. To get further insight into the regulation of Ki67 by D cyclins in our final experiment we used highly proliferating and synchronized quiescent cells. In highly proliferating cells both D cyclins and Ki67 were present at high levels, in contrast to quiescent cells where the expression level of these proteins were low. After releasing cells from cell quiescence we silenced all three D-type cyclins and compared the mRNA expression of Ki67 and the appearance of large multinucleated cells to the highly proliferative and also triple D cyclin silenced cells. Under the later condition when D cyclins were already present in the cells D cyclin silencing did not affect Ki67 expression. In contrast to that, when cells were released from cell quiescence and immediately silenced for D cyclins, the lack of D cyclins in these cells prevented the elevation of Ki67 expression which remained at near background low level suggesting a regulatory connection between D cyclins and Ki67 in HaCaT cells. Moreover, the observation that Ki67 silencing did not affect the expression of D-type cyclins, suggest a downstream position of Ki67 from D cyclins in the regulatory cascade in the cell cycle.

In highly proliferating cultures the number of large multinucleated cells was substantially lower than in triple D cyclin silenced cells released from cell quiescence or in

Ki67 silenced cultures, indicating that the altered cellular morphology may be at least in part the consequence of low Ki67 level.

Finally, we used a mathematical model that puts in context the quantifiably dependence of D cyclins and Ki67 in the formation of multinucleated cells. The fact that not all transfected cells become multinuclear in the culture can be explained by different siRNA transfection efficacy in individual cells within the same culture. Our results suggest that as D cyclin downregulation reaches a threshold, not only cell cycle length but cytokinesis will be affected, thus multinuclear cells will be generated. The transcription factor E2F1 is known to be regulated by D cyclin-Cdk complex [7]. E2F1 plays a role in the late G2 phase by activating Aurora A which is important for centrosomal separation and its inactivation leads to the formation of large multinucleated cells in vitro in HeLa cells [51, 52]. Similarly, according to Burgess et al., in HeLa cells, the aberrant mitosis was due to the failure in chromosome migration to the metaphase plate. These cells showed the same morphological characteristics (cells with multiple or micronuclei) as HaCaT cells in our experiments. In our case the cause of the formation of multinucleated cells may be due to the low level of E2F1 as a result of D cyclin knockdown. The promoter region of Ki67 also contains two E2F-binding sites [27]. Therefore, as E2F1 is a transcriptional activator [53, 54] downregulation of Ki67 may be caused by decreased E2F1 level. Our mathematical model provides a link between D cyclins and Ki67 and gives an explanation to the similar phenotypes in case of silencing. Interestingly, Ki67 and Cyclin D1 expression was higher in chronic psoriatic lesions than in normal epidermis in psoriasis patients [55].

Taken together these data indicate that in some cells the suppression or inhibition of a single D-type cyclin is sufficient to affect cell proliferation, while in others, such as in immortalized, hyperproliferative HaCaT cells, double or triple silencing of D - type cyclins must be applied for influencing cell proliferation and/or morphological changes.

Interestingly, the combined D type cyclin mRNA interference did not result in G0/G1-S block, but in failure of the cytokinesis and the appearance of the large multinucleated cells in the cultures. At a certain threshold value the lack of D cyclins could lead to the dramatic decrease of E2F1 and thus a similar decrease in Ki67 mRNA level. These data suggest that beside their well-known functions during the G0-G1/S phase, D-type cyclins contribute to the regulation of mitosis via influencing Ki67 expression in a downstream manner (**Fig. 7.**)

Conflict of interest

The authors state no conflict of interest.

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Figure and Table legends

Figure 1. The effect of D cyclin silencing on synchronized HaCaT cells.

Contact inhibited and serum starved HaCaT keratinocytes were transfected with cyclin D2 (A), D3 (B) or with the combination of cyclin D1D2, D1D3, D2D3 and D1D2D3 (C) siRNAs and control siRNA and were cultured for 24 hours. mRNA expressions of different types of D cyclins were determined by RT-PCR. Data are indicated as fold change compared to the control siRNA transfected samples. (D) For immunofluorescent detection cells were treated as described above and cultured for 72 hours. Cyclin D1 (green), D2 (pink) and D3 (pink) proteins show distinct nuclear staining (DAPI blue) in the untreated

and control siRNA treated cultures. *(N= 4 independent experiments, data are mean +/- s.d., *p<0.05 determined with Dunnett's test, Scale bar on the images: 100µm).*

Figure 2. Combined silencing of D-type cyclins by siRNAs.

Contact inhibited and serum starved HaCaT cells were nucleofected with the combination of cyclin D1D2, D1D3, D2D3 and D1D2D3 siRNAs and control siRNA and were cultured for 72, 96 and 168 hours after transfection. (A) 72 hours after the combined silencing of D-type cyclins large, multinucleated cells were apparent in the cultures determined by brightfield microscope (A). The percentage of multinucleated cells increased compared to siRNA controls and the highest values observed in the siD1D2D3 nucleofected cell cultures (B). MTT assay was used to measure the cell viability. Data are indicated as absorbance values measured at 540 nm normalized to the mock siRNA nucleofected controls (C). Cell cycle analysis was carried out using PI DNA staining. Data were acquired by a FacsCalibur (BD) and DNA analysis was performed with Modfit software. Percentages of cells in S phase are indicated (D).

*(N= 4 independent experiments, data are mean +/- s.d., *p<0.05 determined with Student T test for cell counting on panel B, with Dunnett's test on MTT assay, Panel D represents one representative measurement, Scale bar on images: 100µm).*

Figure 3. Heatmap of the expressed genes in cyclinD silenced HaCat cells compared to control

Different expression levels of the transcripts of the genes related to cell cycle in siRNA transfected and control HaCat cells. The cluster analysis and dendrogram show the difference between the cell types, and Ki-67 was one of the most significantly changed

genes between the treatments. Green and white colors indicate high and low expression, respectively.

(Values represent the gene expression level of the cell cycle related genes)

Figure 4. The effect of D cyclin silencing on Ki67 expression and the effect of Ki67 silencing

(**A**) Contact inhibited and serum starved HaCaT cells were nucleofected with the combination of cyclin D1D2, D1D3, D2D3 and D1D2D3 siRNAs and siRNA control than were cultured for 24 hours. Ki67 mRNA expressions were determined by RT-PCR. Data are indicated as fold change compared to the siRNA control transfected sample (*) and siD2D3 siRNA transfected sample (**). (**B**) For immunofluorescent detection contact inhibited and serum starved HaCaT cells were nucleofected with the combination of cyclin D1D2D3 siRNAs, siRNA control or left untreated and were cultured for another 72 hours. (**C**) Contact inhibited and serum starved HaCaT keratinocytes were nucleofected with Ki67 and Control for Ki67 siRNAs or with cyclin D1D2D3 and control for siD1D2D3 siRNAs and were cultured for 72 hours. 72 hours after the combined silencing of D-type cyclins or silencing of Ki67 large, multinucleated cells were apparent in the cultures. (D) Contact inhibited and serum starved HaCaT keratinocytes were nucleofected with Ki67 siRNA or siRNA control and were cultured further for 24 hours. mRNA expressions were determined by RT-PCR. Data are indicated as fold change compared to the Control siRNA transfected samples.

*(N= 3 independent experiments, data are mean +/- s.d., *, **$p<0.05$ determined with Dunnett's test, Green: EGFR, Blue: DAPI, Yellow: Ki67, Scale bar on images: 100µm).*

Figure 5. Ki67 expression of 4n G2 cells nucleofected by siRNA control or D1D2D3 cyclin siRNAs

(**A**) Ki67 positive cells were quantified in 4n containing cells nucleofected by siRNA control or D1D2D3 cyclin siRNAs by ScanR high content screening system. The number of Ki67 positive cells decreased in D1D2D3 cyclin silenced cells compared to siRNA control. Slight differences in Ki67 signal (plot heights and positions) could be detected in surface plots between the treatments (**B**) The absolute cell count and the percentage of Ki67 positive 4n cells decreased in D1D2D3 cyclin silenced cell cultures compared to control. (**C**) Representative 100 objects were selected within the 4n gated cell population in siRNA control and in D1D2D3 cyclin silenced cells.

(High content screening was performed in all images, DAPI: blue, Ki67: green, Actin: red on panel A; DAPI: blue, Ki67: green on panel C.

Figure 6. The effect of triple silencing of D cyclins under high Ki67 conditions on HaCaT cells.

(**A**) Cells were grown to 100% confluency. At day 1 following serum depletion Ki67 protein level was high. At day 5 under these conditions Ki67 protein expression decreased dramatically. 24 hours after releasing of these cells from cell quiescence in the presence of serum, cells show a strong Ki67 expression. Flow cytometric analysis was used to detect the expression of Ki67 protein in the cells. FACS profiles of the confluent and subconfluent cells are from one representative experiment. The geometric mean fluorescence intensity (GeoMean) was calculated by subtraction of the GeoMean of isotype-stained cells from the GeoMean of the specific antibody-stained cells using CellQuest Software. (**B**) Group 1 was grown to 70% confluency in FBS containing medium, Group 2 was grown to 100% confluency and serum starved for 5 days. Cells were

nucleofected with D1D2D3 siRNAs and Control siRNA, reseeded at 70% confluency and cultured for 24 hours. Ki67 mRNA expressions were determined by RT-PCR. Data are indicated as fold change compared to the Control siRNA transfected sample. (**C**) Modeling of siRNA effect on G1/S transition. As concentration of siRNA increases, the time needed for G1/S switch (t) increases. Before the switch, pRb level decreases, and E2F1 level increases (a-e). At threshold, the G1/S switch is missing, pRb level remains high and E2F1 level remains low (f). Note that the only the initiation of G1/S transition is modelled, the whole transitional process with resultant protein activity levels in S phase is not simulated.

Figure 7. Schematic model of D-type cyclins contribution in the regulation of mitosis

Table 1. Bioinformatical analysis of multinucleated cells.

Supporting information

Supplementary Figure 1.

(A) APO2.7 – PE staining of double and triple D-type cyclin silenced HaCaT cells**.** Contact inhibited and serum starved HaCaT cells were nucleofected with the combination of cyclin D1D3, D2D3 and D1D2D3 siRNAs and Control siRNA and were cultured for 72 hours. Apoptosis assay was carried out using Apo2.7 PE staining. Data were acquired by a Facscalibur (BD). Flow cytometric analysis revealed no apoptotic effect on these cells. (**B**) Contact inhibited and serum starved HaCaT cells were nucleofected with the combination of D1D2D3 siRNAs and Control siRNA and were cultured for 24 hours. CDC20 mRNA expressions were determined by RT-PCR. Data are indicated as fold expressions compared to the Control siRNA transfected sample from three independent experiments (mean +/- s.d., *P<0.05, Dunnett's test). (**C**) Silencing of Ki67 and D-type cyclins results in decreased protein expression of Ki67. Contact inhibited and serum starved HaCaT keratinocytes were nucleofected with the combination of cyclin D1 D2 D3 siRNAs, Ki67 siRNA and control siRNA (for D type cyclins and for Ki67 separately) and were cultured for 24, 48 and 72 hours. Flow cytometric analysis was used to detect the expression of Ki67 protein in the cells at the indicated times. The geometric mean fluorescence intensity (GeoMean) was calculated by subtraction of the GeoMean of isotype-stained cells from the GeoMean of the specific antibody-stained cells using CellQuest Software and normalized to control siRNA treated cells.

*(N=4 independent experiments on A and B panel, N=1 on C panel, data are mean +/- s.d., *, **p<0.05 determined with Dunnett's test, Scale bar on images: 100µm).*

Supplementary Figure 2.

(A) Dotplots of cells gated for 4n DNA content. (B) Cells double stained for PI and Ki67 showed 1.85±0.49% Ki67 positivity in G2M phase at the quiescent state, which value

continuously increased over the time – 2.30±0.28% at 24h and 21.70±8.63% at 48h – reaching its peak 64.00±3.82% at 72 hour of culture (B)(Data are Mean±SD, percentage of cell in G2/M phase).

Supplementary File. A modified version of the regulatory modules of the mammalian G1/S transition described by Swat et al.

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