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Loss of Par3 leads to aberrant divisions and faster mitotic progression in differentiating primary murine keratinocytes

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída

ABSTRACT

Cell polarity is essential for tissue homeostasis, maintenance of tissue cyto-architecture and other biological processes as oriented cell division, cell adhesion or junction formation. Par3 is a scaffold protein of the Par complex, one of the major polarity complexes conserved in worms, flies and mammals. Loss of Par3 in murine skin epidermis results in the formation of keratoacanthomas, benign keratinocytic neoplasms that commonly arise from UV-exposed areas. Here we show that loss of Par3 in primary murine keratinocytes results in increased aberrant divisions and faster mitotic progression during homeostasis and UV-mediated stress when differentiation was induced. Additionally Par3 deficient keratinocytes show differentiation features even in the absence of a differentiation-inducing stimulus. Our results suggest that Par3 plays a role in mitotic progression and loss of Par3 might predispose primary keratinocytes to an early differentiation phenotype.

RESUMO

Todas as células que constituem o organismo foram geradas através de sucessivas divisões celulares a partir de uma única célula: o ovo fertilizado. Durante o desenvolvimento e estado adulto existe um controlo exato dos processos de divisão, polaridade e destino celular de forma a produzir, controlar e manter os diferentes tipos de tecido que constituem um organismo adulto. Entre estes, o tecido epitelial é de particular interesse pois é responsável pela constituição da maioria dos órgãos nos mamíferos nos mais diversos contextos. Esta multiplicidade implica um leque alargado de funções diferenciadas e extremamente especializadas que só podem ser atingidas através de um grau de organização elevado por partes das células epiteliais de modo a gerar e manter a arquitetura do tecido. Paralelamente, a grande maioria dos cancros humanos provêm de tecidos epiteliais. De facto a perda da polaridade e a desorganização tecidular são pré-requisitos para a formação de tumores epiteliais e sua progressão.

A polaridade apico-basal assenta na ação concertada de proteínas que medeiam a polaridade e que são controladas por sinais externos e internos, tais como factores de crescimento ou sinais do citoesqueleto. Essas proteínas organizam-se em três complexos distintos: Scribbles, Par e Crumbs que foram identificados em invertebrados e que são conservados em mamíferos tanto estrutural como funcionalmente. A disrupção de qualquer um destes complexos já foi demonstrada contribuir para génese de tumores nos mais diversos contextos. Dos três complexos, o PAR tem a função mais alargada, estando envolvido na polaridade apico-basal, polarização neuronal e migração direcionada de células T. O complexo PAR é constituído por duas proteínas Par (*par*titioning defective), Par3 e Par6 com funções estruturais e aPKC (atypical Protein Kinase C), uma serina-treonina cinase. Par3/Par6/aPKC conseguem formar um complexo ternário, no entanto Par3 dissocia-se do mesmo após fosforilação por aPKC. O complexo Par está envolvido em decisões de destino celular pois transmite sinais apicais para regular o alinhamento e orientação do fuso mitótico.

Durante uma divisão celular orientada, a célula tem de alinhar o eixo de divisão paralela ou perpendicularmente em relação ao eixo do tecido. Esta diferente

orientação está acoplada à regulação do destino celular. Se ambas as células filha têm o mesmo destino designa-se por divisão celular simétrica, ou no caso das células filhas terem destinos diferentes é designado por divisão celular assimétrica. A orientação da divisão envolve o alinhamento do fuso mitótico como o eixo de polaridade celular. Proteínas de polaridade como Par3, aPKC e Par6 permitem a transdução de estímulos externos e sinais da maquinaria intracelular para estabelecer a polaridade no interior da célula. Tecidos que proliferam e se autorrenovam mantêm a homeostasia através do rácio entre a manutenção de células estaminais e a geração de células filhas que se comprometem numa via de diferenciação. Sabemos hoje, que as células estaminais podem ser determinantes e até causativas na formação de tumores e que decisões de destino celular defetivas podem contribuir para o comportamento anormal das células estaminais.

A pele é o maior órgão dos mamíferos e representa a primeira linha de defesa do organismo contra agressões externas. É composta por dois compartimentos: um à superfície forrado por epitélio, designado por epiderme e outro mais profundo com propriedades conectivas e de nutrição designado por derme. A epiderme é um epitélio estratificado com capacidade de auto renovação. Numa epiderme normal e saudável, existe um balanço entre a proliferação celular que ocorre na camada basal, a diferenciação das camadas suprabasais e a morte celular. Alterações neste balanço podem resultar em processos de renovação e reparação celular deficientes ou aberrantes que podem em último caso, levar ao envelhecimento da pele, hiperplasias ou até mesmo cancro. Na verdade, a epiderme está em contacto direto com diversos fatores oncogénicos como agentes patogénicos, químicos e radiação ultravioleta (UV) que estão associados ao aparecimento de neoplasias. No entanto uma questão que continua em aberto é como a alteração da polaridade pode alterar este equilíbrio, especialmente através dos processos de divisão celular. Foi anteriormente demonstrado que a perda da proteína Par3 na epiderme murina resultava em fenótipos diferenciados dependendo do contexto. Através de um modelo clássico de indução e promoção da génese de tumores através de DMBA/TPA, a perda de Par3 resultou num menor número e tamanho de papilomas (tumores benignos epiteliais), mediados pela localização errónea da cinase aPKC fora das junções

intercelulares, acompanhados por apoptose e redução da proliferação celular. Estes resultados sugeriram uma função oncogénica para Par3 na epiderme murina. Porém os papilomas que se formaram demonstraram-se muito invasivos. Inesperadamente a perda de Par3 também predispôs os animais para a formação de queratoacantoma, um tipo de neoplasia muito rara em *M. musculus* mas frequentemente observadas em humanos. Desta forma Par3 atua na epiderme murina tanto como oncogene ou gene supressor de tumores dependendo do contexto. Considerando o papel do complexo Par no destino celular e génese de tumores, o objectivo deste trabalho é estudar o efeito da perda de Par3 sobre a divisão celular e o seu contributo para a génese de tumores em queratinócitos epidérmicos murinos durante homeostasia e *stress* mediado por radiação ultravioleta.

Neste estudo observámos que a perda de Par3 em queratinócitos primários predispôs para divisões celulares aberrantes em condições de diferenciação. Paralelamente, os queratinócitos Par3 KO que progrediram através de uma divisão celular correta, demonstraram passar menos tempo em mitose que os controlos, sugerindo uma progressão mitótica mais rápida. Demonstrámos igualmente que a indução da diferenciação leva a amplificação de centrossomas e aneuploidia *in vitro* mas independentemente do genótipo. Observou-se também que, em condições de não diferenciação a perda de Par3 levou ao aparecimento de fuso mitóticos multipolares. De seguida foi avaliado o ciclo celular nas duas populações de queratinócitos: as célulasPar3 KO demonstraram um perfil de FACS comparável ao dos controlos aquando da diferenciação, sugerindo um perfil de diferenciação precoce. A perda de Par3 predispôs as células primárias para uma mais rápida progressão mitótica após irradiação por UV. Ao analisarmos a área do núcleo, as células Par3KO demonstraram ter uma maior área nuclear quando comparadas com os controlos nas condições de não diferenciação. Analisou-se a resposta ao dano no ADN através do marcador 53BP1. Em condições de não diferenciação após irradiação das células, os queratinócitos Par3 KO demostraram responder ao dano no ADN de forma diferenciada quando comparados com os controlos. Nas secções de queratoacantomas das nossas experiências *in vivo* observámos divisões celulares assimétricas, simétricas e aberrantes.

Este estudo aponta para uma possível nova função de Par3 na regulação da progressão mitótica durante a homeostasia e *stress* induzido por radiação UV. Os queratoacantomas são tumores de crescimento rápido que advêm de áreas da pele exposta à radiação solar, no entanto a sua etiologia é pouco conhecida. As nossas observações contribuem para a compreensão do impacto da perda de polaridade na homeostasia tegumentar e abrem possíveis vias para o entendimento da génese dos queratoacantomas.

INTRODUCTION

An adult human body comprises approximately 10^{13} cells. All of these cells have been generated through successive cell divisions starting with one single cell: the fertilized egg. During development and adulthood, an exact control of the cell division, cell polarity and cell fate processes must exist in order to produce, control and maintain the several types of tissues that constitute an adult organism (Noatynska et al. 2013). Among this the epithelium is of particular interest by being responsible for the constitution of the majority of the organs in the mammalian body (McCaffrey & Macara 2011). This multiplicity implicates specialized and differential functions that are achieved by distinct organization of the epithelial cells (Royer & Lu 2011). Interestingly, most of the human cancers arise from epithelia (McCaffrey & Macara 2011; Royer & Lu 2011) and in fact loss of polarity and tissue disorganization is a prerequisite for epithelial tumor formation (Iden et al. 2012) and progression (Macara et al. 2013).

Cell polarity can be defined as the uneven distribution of cellular constituents such as proteins, RNAs and lipids in order to establish asymmetry at both structural and functional levels (Vorhagen & Niessen 2014). In the epithelial context, different types of polarity are discriminated: planar cell polarity refers to asymmetry in the plane of an epithelium, whereas apico-basal polarity refers to polarized structures in a cell perpendicular to the epithelial sheet (Rodriguez-Boulan & Macara 2014). The establishment of apico-basal asymmetry culminates eventually in two biochemically and structurally different domains: the apical and basolateral domains. These compartments have defined protein and lipid content and are physically delimited by adherens (AJ) and tight junctions (TJ). The AJ mediate strong physical associations between cells, and their disruption leads to looseness of cell-cell contacts and consequent disorganization of tissue cytoarchitecture (Meng & Takeichi 2009). The TJ are part of a continuous intercellular barrier among the epithelial cells .and regulate the passage of solutes and cells across the intercellular space. TJ make semi-permeable barrier with permselective capability which regulates the movement of solutes, ions or even cells depending on their size and charge. In addition TJ act as a fence(Steed et al. 2010). TJ are structures composed of several tight junction specific proteins such as claudins, occludins and junction adhesion molecules (JAMs) that allow

TJ to be dynamic structures with both barrier and regulatory functions (Steed et al. 2010). Interestingly TJ participate in apico-basal polarity,-in contrast to a non-polarized cell where the plasma membrane constituents can shuffle and mix continuously, in apico-basal polarized cell they are confined to a distinct region defined by the junctional complexes (Sabherwal & Papalopulu 2012; Khursheed & Bashyam 2014).

Apico-basal polarity relies on concerted action of polarity proteins that are controlled by extrinsic and intrinsic cues such as growth factor gradients or the cytoskeleton (Iden & Collard 2008). These key proteins assemble to three distinct complexes: Scribbles, Par and Crumbs complexes that have been identified in invertebrates and are highly conserved in mammals both structurally and functionally (Assémat et al. 2008). The Scribble complex defines the basolateral domain and Crumbs and Par complexes define the apical domain and the apico-lateral border, respectively (Aranda et al. 2008). Interestingly, the disruption of any of these complexes has been shown to contribute to tumorigenesis in different contexts. Mutations in Scribble complex genes result in loss of apico-basal polarity and induction of cell overproliferation with malignant-like characteristics when combined with *Ras* mutations in *D. melanogaster* (Bilder 2004). In mice specific loss of SCRIB in prostate in combination with *Ras* mutation results in promotion of cancer progression and recapitulates the human disease. In addition SCRIB deregulation was correlated with poor prognosis in human prostate cancer in the same elegant study (Pearson et al. 2011). C*rumbs3,* a transmembrane protein and member of the Crumbs complex, was demonstrated to suppress epithelial tumor progression *in vitro* (Karp et al. 2009) and Crumbs was demonstrated to act as tumor suppressor in *D. melanogaster* (Ling et al. 2010). Par complex proteins were shown to be upregulated in human carcinomas (Nolan et al. 2008; Huang & Muthuswamy 2010) and to be involved in breast tumorigenesis and progression (McCaffrey et al. 2012; Xue et al. 2012), prostate cancer progression (Zhang et al. 2015) and mouse skin tumorigenesis and invasion (Iden et al. 2012).

From the three complexes the Par complex is the one with more ubiquitous function by being involved not only in apico-basal polarity but also in neuronal polarization, directed cell migration and T cell polarization (Macara 2004; Aranda

et al. 2008). The PAR complex is constituted by two Par (*par*titioning defective) proteins, Par3 and Par6 - two multidomain scaffold proteins - and aPKC (atypical Protein Kinase C), a serine-threonine kinase. Par3/Par6/aPKC can form a ternary complex, however, Par3 can dissociate from the complex in an aPKC-phosphorylation dependent manner. Upon upstream activation, the small GTPase Cdc42 can associate with Par6, which leads to the activation of aPKC and results in the dissociation of Par3 from the complex (Joberty et al. 2000; Horikoshi et al. 2009; Morais-de-Sá et al. 2010). Par3 by itself is capable to interact with several adhesion molecules such as p57, Nectin and tight junction molecules like Junction Adhesion Molecules (JAMs) and Tiam1/2 (Chen & Zhang 2013; Chen & Macara 2005).

The Par complex proteins are also involved in cell fate decisions by transmitting apical polarity cues to regulate mitotic spindle alignment and spindle orientation (Williams & Fuchs 2013; Vorhagen & Niessen 2014). During an oriented cell division a cell aligns its division axis either perpendicular or planar to the tissue axis. In fact this differential orientation is frequently coupled to cell fate regulation. If both daughter cells have the same fate this is designated as symmetric cell division (SCD), whereas if daughter cells adopt different fates this is referred to as asymmetric cell division (ACD). The division orientation involves the alignment of the spindle to the cell polarity axis. Polarity proteins Par3, aPKC and Par6 can transduce external stimuli and signals to the intracellular machinery to establish the intracellular polarization. Furthermore aPKC and/or Par3 control spindle orientation and cell fate decisions during murine mammary gland development, in line with the observations in *C. elegans* and *D. melanogaster* (Vorhagen & Niessen 2014). Tissues that proliferate (and self-renew) maintain homeostasis through a concerted ratio between stem cell maintenance and the generation of daughter cells that undergo differentiation. Interestingly we know nowadays, that stem cells can be causative in terms of tumor formation and that defective cell fate decisions can contribute to an abnormal behavior of stem cells (Mescher & Iden 2015) .

The skin is the biggest organ in mammals and it represents the first line of defense of the organism against external factors. The skin is composed of two compartments: an inner structure with connective and nutritive properties- the

dermis and an epithelial component that coats the entire skin surface-the epidermis (Baroni et al. 2012).

The epidermis is a self-renewing stratified epithelium. Just above the dermis is the basal layer, followed by the spinous layer, granular layer and on the top, the cornified layer. In general the epidermis serves a barrier function by preventing water loss and defining both the inside and outside borders of the organism. Whereas the cornified layer provides the major physical barrier and barrier forming lipids produced by outermost viable cells, the underlying layers contain important cell-cell junctions and cytoskeletal-associated proteins (Proksch et al. 2008). The basal layer retains the renewal capability and is responsible for epidermal cellular turnover (Blanpain & Fuchs 2006). In the epidermis the major cell type is the keratinocyte. From the basal layer until the granular layer, keratinocytes are nucleated, viable and progressively mature and differentiate when passing the different layers. However, in last steps of their differentiation process, keratinocytes lose their nuclei and undergo strong structural alterations to form the cornified layer (Baroni et al. 2012).

The skin epidermis exhibits polarity in a different sense when compared to classical epithelium like in intestine. The latter has clear apical and basolateral domains defined by an apical junctional complex. In the skin, the different epidermal layers have a distinct set of differentiation and junctional markers (Helfrich et al. 2007). It is known already that polarity proteins differentially localize in various layers and their expression in part differs (Niessen et al. 2012).

In normal and healthy epidermis, a balance between proliferation that endures in the basal layer, differentiation of the suprabasal layers, and cell death exists. It has been reported previously that one of the mechanisms of epidermal stratification and differentiation is through asymmetric cell division (Lechler & Fuchs 2005). We know nowadays that the Par complex proteins are involved in this equilibrium. In the mammalian system loss of aPKClambda results in altered epidermal homeostasis, differentiation and increased asymmetric cell divisions (Niessen et al. 2013). Interestingly loss Par3 in embryonic epidermis leads to randomized spindle orientation (Williams et al. 2014). Alterations in the balance of oriented divisions can result in impaired renewal and repair and subsequent

hyperplastic growth and cancer, or skin ageing (Baroni et al. 2012; Mescher & Iden 2015). In fact the epidermis is in direct contact with several oncogenic factors as pathogens, chemicals and UV radiation that can lead to cellular overgrowth and neoplasia. An open question still remains as how impaired polarity can impact this balance, especially cell division processes. Previously, it has been shown that deletion of the Par3 polarity protein in murine epidermis had highly context dependent phenotypes. Using a classical DMBA/TPA skin tumorigenesis experiment, loss of Par3 resulted in reduced number and size of papillomas, mediated by mislocalization of aPKC away from the cell-cell junctions, accompanied by increased apoptosis and reduced cell proliferation. These results suggested a tumor promoting function of Par3 in mouse epidermis. On the other hand the papillomas that had formed in the Par3 epidermal KO mouse skin were highly invasive and the mice developed as well keratoacanthomas, a very rare but aggressively growing cancer type in mice but frequently formed in humans and therefore clinically relevant. Strikingly, Par3 in murine epidermis therefore acts both as tumor promoter and tumor suppressor depending on the context (Iden et al. 2012).

Taking into consideration the role of the Par complex in cell fate (Niessen et al. 2012) and skin tumorigenesis (Iden et al. 2012), we wanted to address how loss of Par3 can impact cell division and contribute to tumorigenic processes in skin keratinocytes during homeostasis and UV-mediated stress conditions.

RESULTS

Loss of Par3 accelerates mitotic progression and results in aberrant divisions

To assess how the loss of Par3 can impact cell division processes, we analyzed by live cell imaging primary murine keratinocytes freshly isolated from newborn K14Cre;Par3fl/fl;H2B-GFP mice. These cells constitutively express Green Fluorescent Protein (GFP) fused to the DNA-binding histone 2 B (H2B), therefore labelling the nucleus. As the skin epidermis is constituted by undifferentiated and differentiated cells, we have analyzed both control and Par3 KO populations at undifferentiating and differentiating conditions. For that, one day after seeding, half of the cultures were switched from Low Calcium concentration medium (50µM Ca^{2+}, LC), to a High Calcium concentration medium (1,8 mM Ca^{2+}, HC). This process termed Calcium Switch (CS) induces cadherin-mediated intercellular adhesion, i.e. allows tight and adherent junctions' formation. We started live cell imaging 36 hours after CS and followed the primary cultures for another 12-24 hours. The onset of differentiation eventually induces cell cycle arrest. Therefore, we chose a relatively early time after CS during which cells are still dividing. Interestingly, when Par3 KO primary keratinocytes were cultured at HC conditions, they divided aberrantly more frequently (32% of divisions) when compared to the controls (7% of the divisions) (Figure 1A, Movie M2/4), suggesting that Par3 could be important for proper cell division in murine keratinocytes. We next assessed if cell division kinetics were altered by loss of Par3. While both at LC and at HC conditions, aberrantly dividing Par3 KO cells exhibited a mitosis duration comparable to control cells, the population of Par3 KO cells that divided correctly accomplished mitosis in shorter time (mean mitosis duration 52,03 minutes, controls: 57,82 minutes) (Figure 1B). Together these results suggest that loss of an important polarity regulator like Par3 predisposes to division aberrancies and a faster mitotic progression, and open the possibility that polarity signaling contributes to cell cycle checkpoint control.

We further investigated mitosis kinetics in SV40-immortalized control and Par3-deficient keratinocyte cell lines, therefore in a cellular context already more susceptible for incorrect divisions.

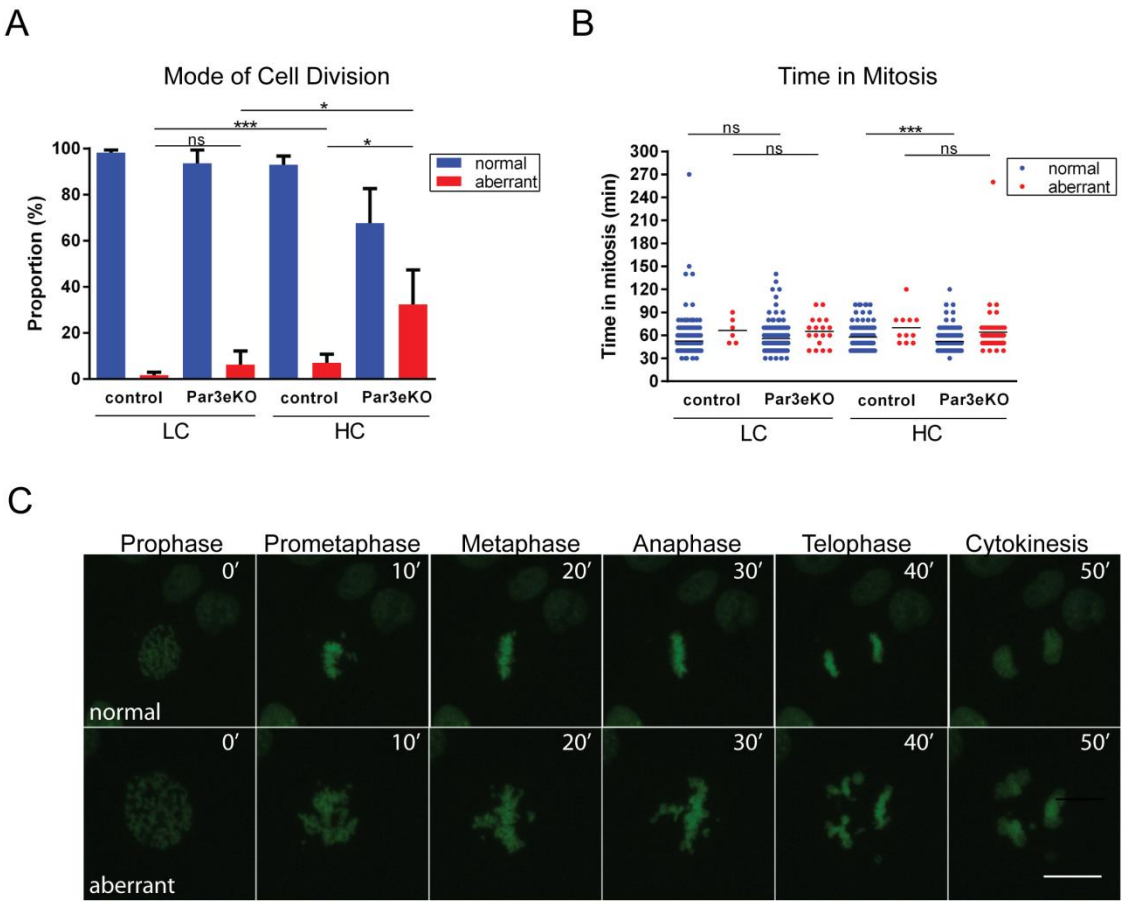

Figure 1. Shorter Mitosis Duration and Increased Aberrant Divisions in Par3-Deficient Keratinocytes

(A) Quantification of normal and aberrant divisions (n=3), bars represent mean±SD; ns: $p > 0.05$; *: $p \leq 0.05$; ***: $p \leq 0.001$.

(B) Quantification of mitosis duration, bars represent mean. Significance was evaluated using a nonlinear mixed-model (detailed in Material and Methods) (n=3); ns: $p > 0.05$; ***: $p \leq 0.001$.

(C) Representative micrographs of normal and aberrant divisions, top right numbers represent time in minutes, scale bar, 50 µm.

SV40 causes inactivation of the tumor suppressor protein p53, and consequently these keratinocytes lack an important mitotic checkpoint component and keeper of genome integrity. In fact loss of Par3 similarly resulted in more aberrant divisions when compared to the controls: Of all dividing cells, Par3 KO keratinocytes exhibited 15% of abnormal divisions, whereas in control keratinocytes 7% abnormal divisions were observed at HC conditions (Figure S1A, Movie M6/8). This therefore confirmed our results obtained with primary keratinocytes, though with smaller effects. Together we show here evidences that

Par3 contributes to proper mitosis duration and cell division in murine epidermal keratinocytes in vitro.

These observations were especially pronounced at LC conditions, hence when the Par complex does not localize to the junctions and by that means, cannot assemble important downstream signaling cascades required for proper junction maturation and establishing of the polarity.

Par3 deficiency does not result in centrosome amplification and aneuploidy

Aberrant divisions can be caused by various defects or perturbations, such as for instance centrosome amplification. To examine if increased aberrant divisions in Par3 deficient keratinocytes were a consequence of centrosome amplification, we performed immunocytochemistry of the centrosome markers, γ-tubulin and pericentrin. Throughout one cell cycle, the centrosome undergoes dynamic changes in terms of subcellular localization and molecular composition. Using above mentioned markers, in G0/G1 phase a single structure is visible as result of proper centrosome segregation during mitosis. At S phase centrosome duplication takes place, giving rise to two adjacent centrosomes. From late G2 phase onwards until the M phase the two centrosomes translocate and reposition to opposing poles of the nucleus, resulting in two distant dot-like signals that later typically serve to nucleate the mitotic spindles. If any of these processes is disturbed or delayed, there is increased risk of centrosome amplification - a common feature in cancer cells. Surprisingly, loss of Par3 did not significantly alter centrosome numbers, suggesting that the increased number of centrosomes was not causative for the aberrant divisions observed in Par3 KO keratinocytes (Figure 2A,B). Similarly, loss of Par3 did not result in altered centrosome amplification in spontaneously immortalized cells at higher passages (p88-90) suggesting that the increased divisions' phenotype was not directly centrosome-related (Figure S2A,B).

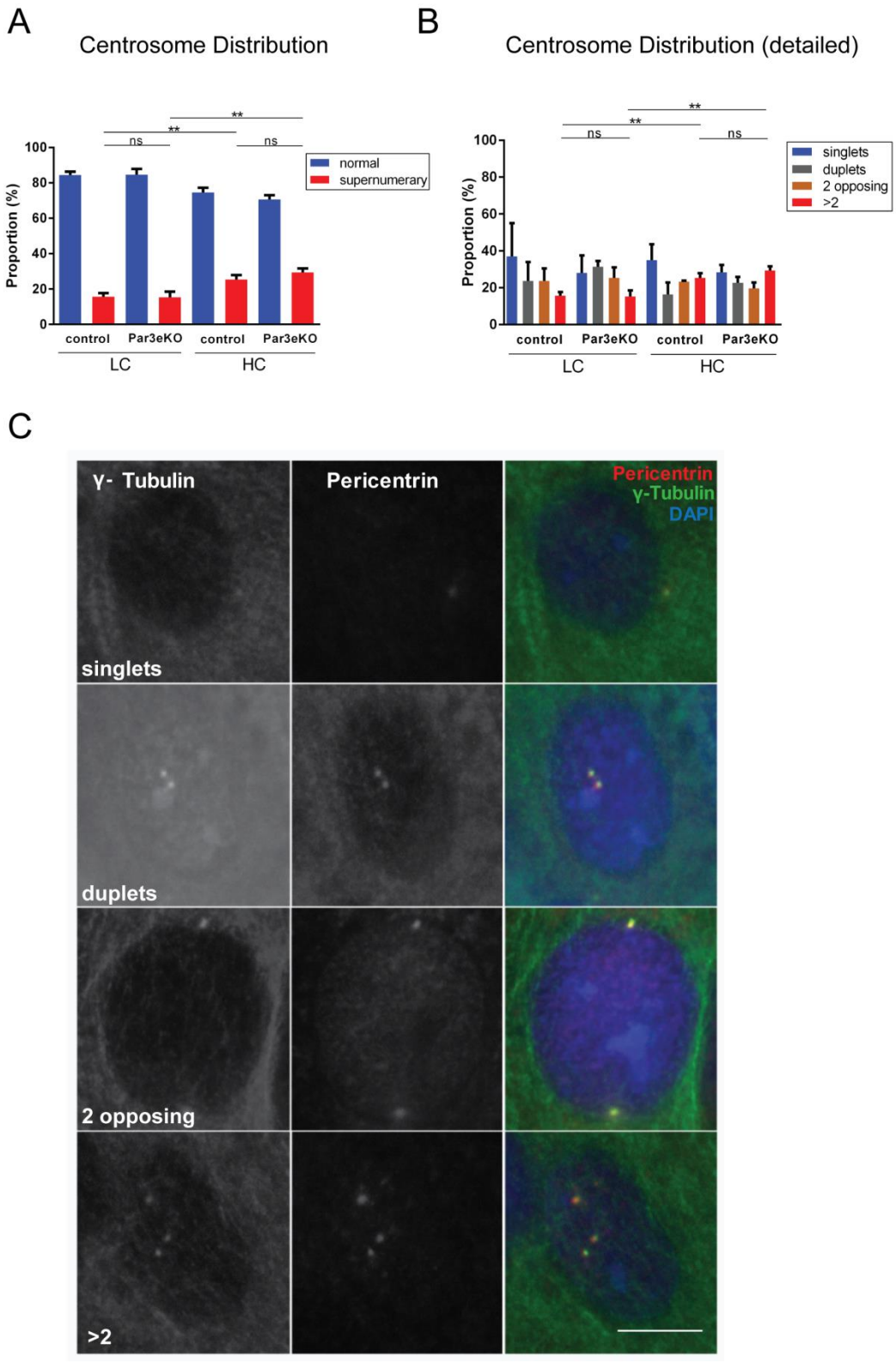

Figure 2. Par3 Deficiency Does Not Lead to Centrosome Amplification in Primary Keratinocytes

(A) Quantification of cells with normal and supernumerary centrosomes (n=3), bars represent mean±SD; ns: $p > 0.05$; **: $p \leq 0.01$.
(B) Quantification of centrosome numbers and positioning (n=3), bars represent mean±SD.
(C) Representative micrographs of the four modalities of centrosome categorization: singlets, duplets, 2 opposing and >2, scale bar, 20 µm.

We further investigated the contribution of Par3 to cell aneuploidy, chromosomal aberrations that result from unequal chromosome segregation during cell division and that are frequently observed in cancer cells. In immunocytochemistry studies, nuclei were visualized using DAPI to evaluate the number of aneuploid cells. We considered cells as *aneuploid* when they were multinucleated, possessed an unusual shape or when they showed nuclear extrusions. Interestingly, quantification of these phenotypes revealed that Par3 deficiency under the conditions tested did not predispose to aneuploidy in primary keratinocytes (Figure 3A, B). Similar results were obtained using spontaneously immortalized cells at higher passages (p88-90) (Figure S2C,D). Interestingly, we detected a higher correlation between centrosome numbers and aneuploidy at HC conditions. The induction of differentiation thus seems to promote centrosome amplification that can be causative of the aneuploidy, independently of Par3.

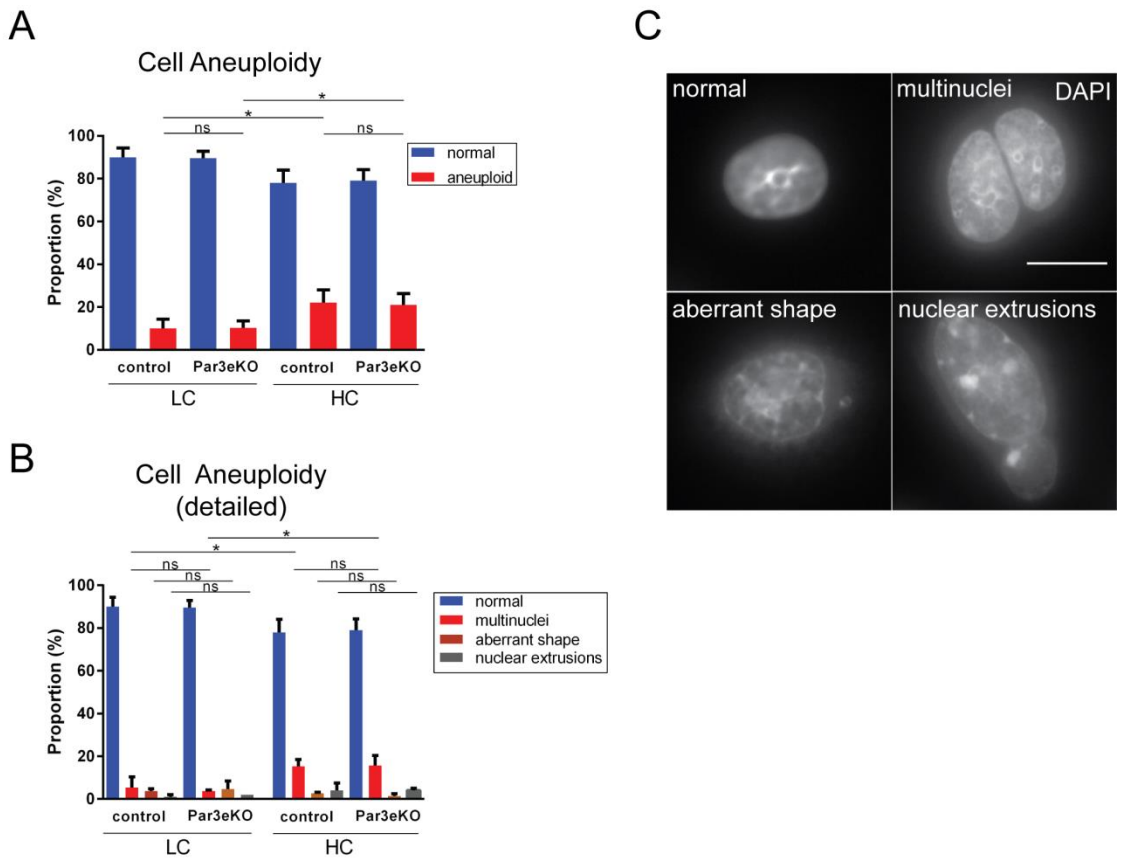

Figure 3. Loss of Par3 Does Not Result in Cell Aneuploidy in Primary Keratinocytes

(A) Quantification of cell aneuploidy (n=3), bars represent mean±SD; ns: $p > 0.05$; *: $p \leq 0.05$.
(B) Quantification of nuclear phenotypes (n=3), bars represent mean±SD; ns: $p > 0.05$; *: $p \leq 0.05$.

Par3 deficiency predisposes to spindle abnormalities at undifferentiated conditions

Centrosome amplification is often causative of aneuploidy and can drive the onset of aberrant divisions; however, centrosomes do not always nucleate the mitotic spindle as demonstrated by the elegant work of Terry Lechler (Sumigray et al. 2011; Sumigray & Lechler 2011). Furthermore, when we previously focused on the centrosome numbers, we considered all cell populations, both dividing and quiescent. In order to assess specifically the dividing population we performed immunocytochemistry of both centrosome (pericentrin) and spindle (α-tubulin) markers in primary keratinocytes at undifferentiated (LC) and differentiated (HC) conditions, to identify cells undergoing mitosis. Surprisingly we found a robust induction of multipolar spindles at LC conditions upon loss of Par3 (Figure 4A), suggesting that predisposition for aberrant divisions is already present without differentiation stimuli. However, in contrast to our previous findings obtained by live cell imaging of fluorescently labeled nuclei (Figure 1A) we couldn't detect any significant difference between control and Par3 KO keratinocytes at differentiation conditions.

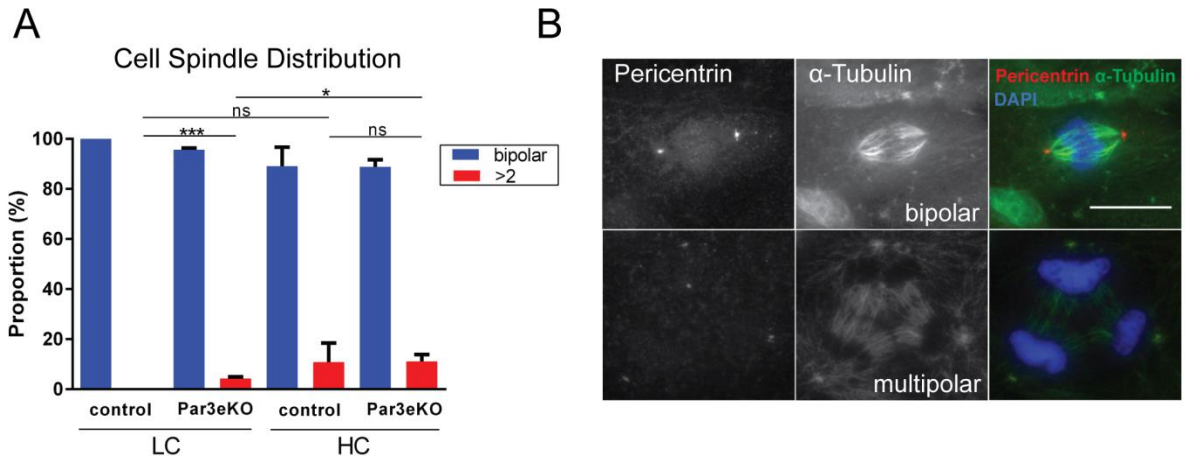

Figure 4. Par3 Deficient Keratinocytes Exhibit Spindle Aberrancies at Undifferentiated Conditions

(A) Quantification of number of cells with bipolar and multipolar spindles (n=3), bars represent mean±SD; ns: $p > 0.05$; *: $p \leq 0.05$; ***: $p \leq 0.001$.
(B) Representative micrographs of cells with bipolar (top) and multipolar (bottom) spindles, scale bar, 20 μm

Early differentiation cell cycle profile in immortalized Par3 KO keratinocytes

Since Par3 KO keratinocytes are predisposed to aberrant divisions and faster mitotic progression, we wanted to address the effect of loss of Par3 on overall cell cycle. Additionally we wanted to assess if the aberrancies that we described and that are Par3-dependent (aberrant divisions and LC multipolar spindles) and Par3-independent (centrosome amplification, aneuploidy and HC multipolar spindles) could lead to DNA content alterations. We used spontaneously immortalized keratinocytes of higher passages (P92-94) and performed Cell Cycle FACS DNA content analysis. Using this type of cells conferred us several technical advantages: a more robust and homogenous cell tool compared to the primary keratinocytes, with higher chances for cell cycle synchronization and incommensurable availability. Cell cycle synchronization in murine keratinocytes is technically difficult and never complete. We therefore serum starved cultures for 36 hours in order to get at least qualitatively more homogeneous populations. At LC conditions control keratinocytes exhibited a balanced ratio of cell populations in G0/G1 and S phase and cells that undergo G2/M phase. In contrast, Par3 KO cultures showed a smaller G2/M population and increased S phase population compared to control cells. Surprisingly at HC conditions both control and Par3 KO populations exhibited a profile that resembles the Par3 KO profile at LC conditions (Figure 5A). Surprisingly none of our reported phenotypes contributed to detectable abnormal haploid or polyploid populations. These results indicate that in terms of cell cycle control Par3 KO keratinocytes prematurely differentiate even in the absence of differentiation-inducing stimuli like extracellular calcium.

Loss of Par3 results in shorter mitosis duration and altered 53BP1 foci upon UV-mediated stress

In order to assess the impact of loss of polarity protein function on the mitosis progression upon UV irradiation, we performed live cell imaging of primary murine keratinocytes freshly isolated from newborn K14Cre;Par3fl/fl;H2B-GFP mice. One day after seeding, half of the cultures were switched to HC medium.

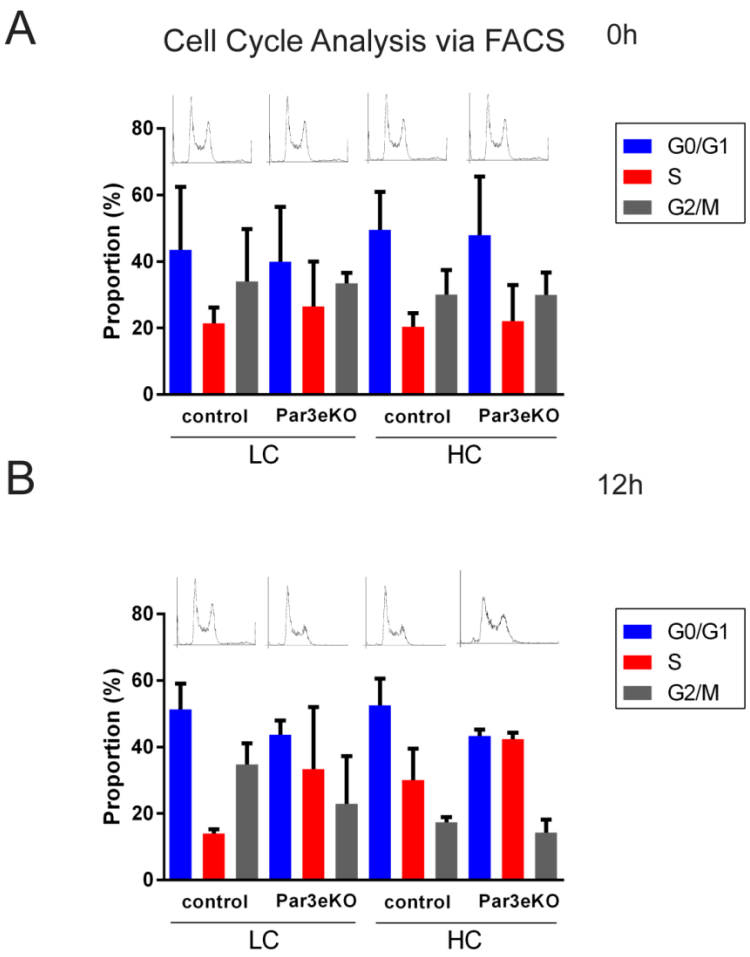

Figure 5. Loss of Par3 Leads to Cell Cycle Signature of Differentiated Cells Spontaneous Immortalized Keratinocytes

(A) Overall distribution of cell cycles phases, at starved conditions (0h) and just after release (12h) (n=2), bars represent mean±SD.

Cultures were irradiated using 5 mJ/cm^2 dose of UVB 24hours after the CS and we started the imaging 12 later (36 hours CS). Previously, it has been described that UV irradiation induces both G1 and G2 checkpoint cell cycle arrest in human keratinocytes (Pavey et al. 2001). In our system the same was noted: As result of the UVB induced cell cycle arrest fewer division events and increased apoptosis but also reduced cell motility were observed. As described before (Figure 1B), under differentiating conditions Par3 KO keratinocytes divide faster (53,33 minutes) than the controls (60,66 minutes) without UV exposure (Figure 6A). Interestingly, whereas UV irradiation resulted in overall decelerated mitotic

duration of control cells (65,71min), Par3-deficient keratinocytes retained a faster mitotic progression profile (55,26 minutes) (Figure 6A).

These results highlight that Par3 controls mitosis progression both during homeostasis and upon UV-mediated stress, and suggest differential response to UV-induced damage when this polarity protein is lost. Paradoxically, work in the Iden laboratory revealed that loss of Par3 in a UV-mediated stress context leads to ectopic activation of checkpoint proteins ATR and Chk1 (Letzian et al., unpublished). On the one hand the activation of ATR-damage sensing pathway could explain a pre-selection of cells with less damage (and not arrested) that excel as faster mitotic cells, on the other hand interesting new findings from other groups can widen the way we look at it. In fact the overexpression of Chk1 is common in several tumor types. Whereas Chk1 has previously been considered a tumor-suppressor, this view has meantime changed. Studies in Acute Lymphoblastic Leukemia related Chk1 overexpression with proliferation and survival, mostly by preventing apoptosis and excessive replicative stress (Sarmento et al. 2014). We speculate that Chk1 overactivation in Par3 KO could protect the keratinocytes from mitotic catastrophe in a UV-dependent manner; however, additional functional studies are required to address this question. We wondered if the faster mitosis progression in Par3 KO keratinocytes correlates with elevated Chk1 levels.

From above experiments, we further tested if the nucleus area was altered upon Par3 dysfunction. Using an automated, unbiased image quantification software (Cell Profiler, Broad Institute, Cambridge) the average nucleus area was analyzed (Figure 6B). In general keratinocytes nuclei area at HC conditions was bigger than in LC. However, when the cells were not irradiated no major differences were found between control and Par3 KO keratinocytes independently of the extracellular calcium level. Upon UV irradiation Par3 KO nuclei were significantly bigger than the control cell nuclei at LC conditions but not when differentiation was induced (Figure 6C). These results suggest that the area of the nuclei increases upon differentiation independent of UV irradiation. However upon UV irradiation Par3 KO keratinocytes were bigger than the controls at undifferentiating conditions, suggesting an early nuclei area induction upon UV stress.

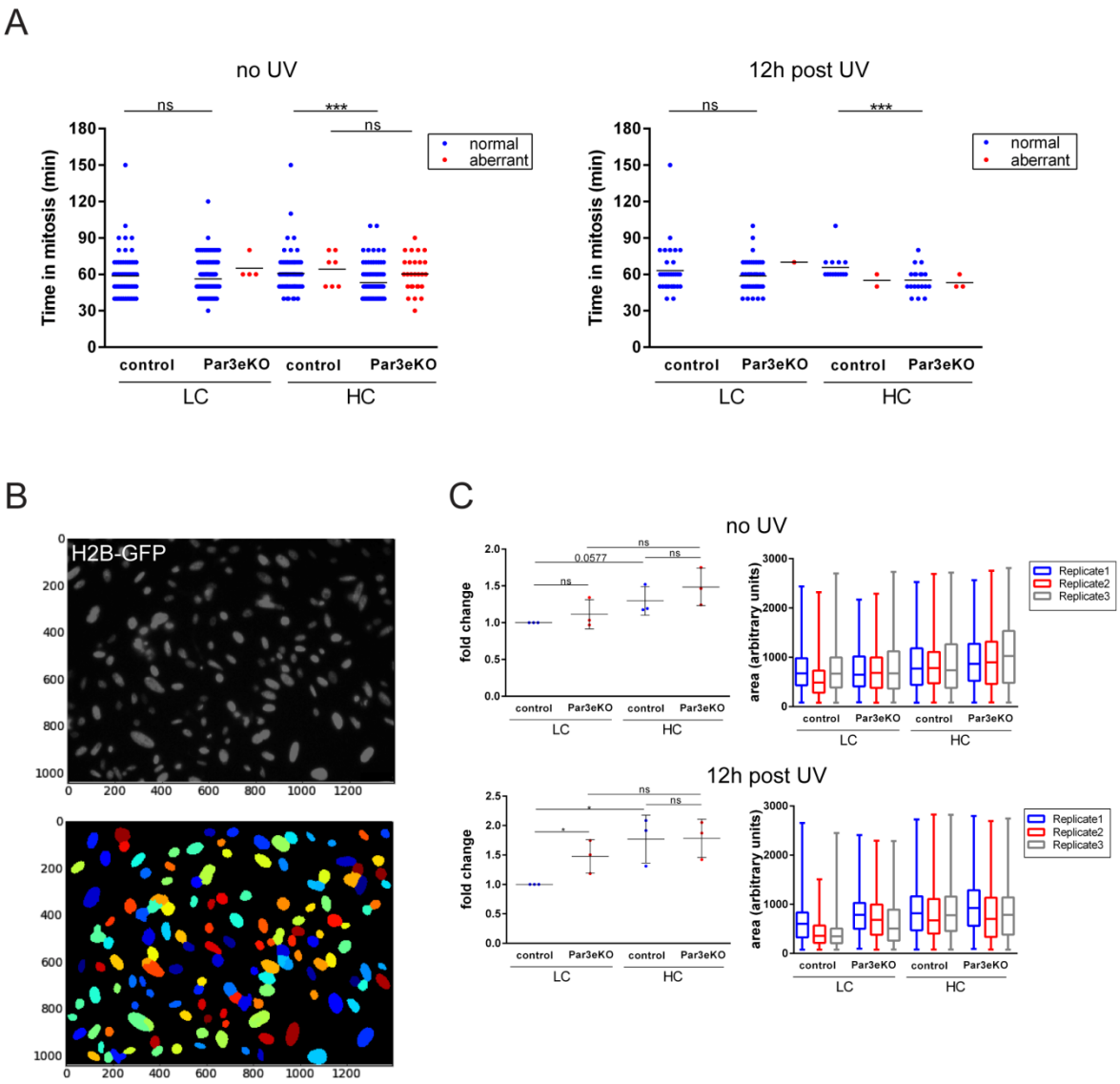

Figure 6. Shorter Mitosis Duration Upon UV-mediated Stress in Par3 KO Keratinocytes

(A) Quantification of mitosis duration: no UV (left), UV (right), bars represent mean. Significance was evaluated using a nonlinear mixed-model (Detailed in Material and Methods) (n=3); ns: $p > 0.05$; ***: $p \leq 0.001$.

(B) Representative Cell Profiler workflow images, GFP channel (top), Primary Objects Identification (bottom).

(C) Quantification of cell area, no UV (top), UV (bottom) (n=3), bars represent mean±SD; ns: $p > 0.05$; *: $p \leq 0.05$. In the right overall distribution among the three replicates, no UV (top), UV (bottom).

We further wanted to understand if Par3 KO keratinocytes recover differentially from UV-induced damage when compared to controls. To address this question we irradiated freshly isolated murine keratinocytes (K14Cre;Par3fl/fl;H2B-GFP) and subsequently performed immunocytochemistry for 53BP1 (53 binding protein-1).

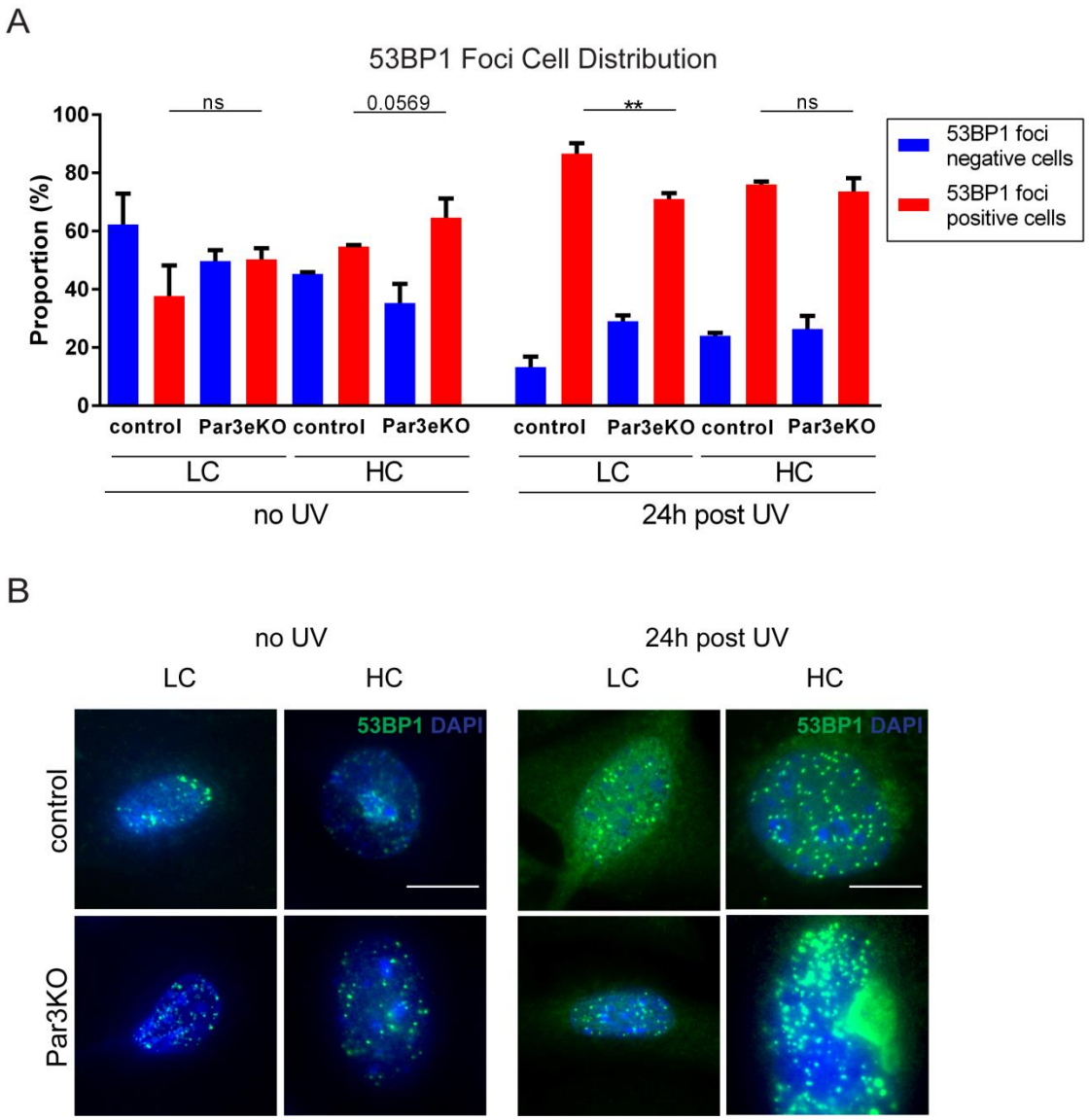

Figure 7. Altered 53BP1 Foci in Par3 KO Keratinocytes Upon UV-mediated Stress at Undifferentiated Conditions

(A) Quantification of positive and negative foci cells without UV and upon UV irradiation (n=3), bars represent mean±SD; ns: $p > 0.05$; **: $p \leq 0.01$.
(B) Representative micrographs of foci negative (left) and positive (right) cells, scale bar, 20 µm.

This marker of early DNA damage lesions is a chromatin-associated factor that is recruited to UV-induced lesions to DNA strand breaks. Upon UV irradiation, 53BP1 accumulates at sites close to DNA lesions. The cells were irradiated with a dose of 5mJ/cm^2 and fixed 24 hours after irradiation. Interestingly also in non–irradiated cells, 53BP1 foci were detectable in both control and Par3 KO keratinocytes, though with strongly reduced overall intensity, suggesting that both control and Par3 KO populations were actively sensing damage and

consequently undergoing genomic stress. Surprisingly, despite more foci in Par3 KO keratinocytes at LC conditions before irradiation, 24 hours after UV-B irradiation, Par3 KO cells at LC conditions had less foci cells than controls (Figure 7A,B). Upon differentiation loss of Par3 had no effect on 53BP1 foci numbers (Figure 7A,B). Together these results suggest that Par3 deficient keratinocytes show a differential response to UV induced damage, as evidenced by the activation of 53BP1 at undifferentiated conditions. However, to assess if the response differences between Par3 KO and control keratinocytes directly correlate with differential damage recovery, more careful kinetic studies are required.

Asymmetric, Symmetric and Aberrant Divisions in Par3 KO Keratoacanthomas

Finally we asked if aberrant division could also be observed in different skin tumors of Par3 eKO mice *in vivo*, as upon carcinogen treatment loss of Par3 resulted in altered skin tumorigenesis (Iden et al., 2012).

In order to assess the mode of division we performed immunohistochemistry of tumor cross sections of keratoacanthomas formed in adult K14Cre;Par3fl/fl mice. We stained for pericentrin and α-tubulin to mark centrosomes and mitotic spindles, respectively. We focused on the basal layer that in this type of tumors maintains the overall cytoarchitecture and tissue organization. Indeed, in the specimen analyzed, we observed asymmetric (Figure 8A) and symmetric (Figure 8A) and aberrant divisions (Figure 8A). Interestingly, the latter was mostly noted in areas where focal invasion seemed to occur. However, co-immunostaining with markers for epidermal-dermal junction or basement membranes, like collagen IV, laminin-332 or beta 4 integrins as well as more extensive analysis of additional samples and quantification would be necessary to draw a firm conclusion on this.

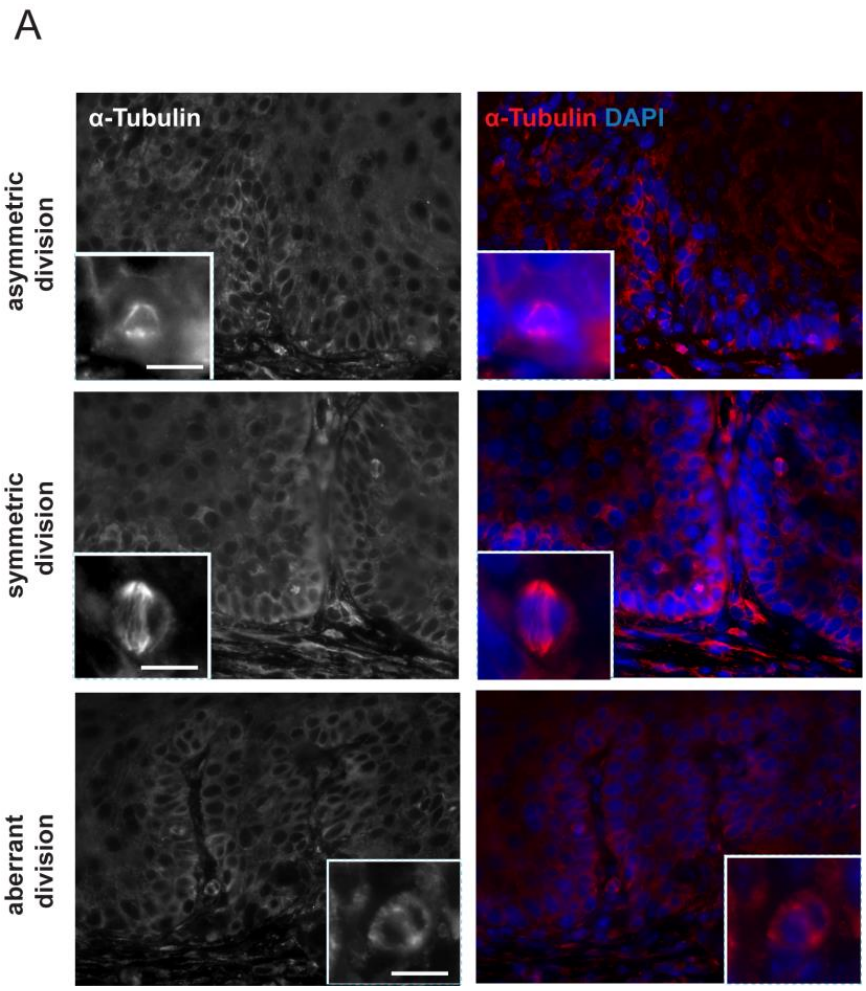

Figure 8. Asymmetric, Symmetric and Aberrant Divisions in Par3 eKO Keratoacanthomas

(A) Representative micrograph of keratoacanthoma basal layer and asymmetric cell division, symmetric cell division and aberrant division, scale bar, 20 μm

DISCUSSION

This study reveals a possible function of Par3 in mitotic control and in cell division processes in primary keratinocytes. Previously the Par complex has been implicated in cell division orientation, cell fate decisions (Niessen et al. 2013; Williams et al. 2014; Hao et al. 2010; Bultje et al. 2010) and skin tumorigenesis (Iden et al. 2012). However any association between cell cycle and Par3 was unknown. We describe here a possible role of Par3 during mitosis in murine epidermal cells. Par3 deficient keratinocytes divide aberrantly more frequently when compared to the controls at differentiated conditions.

Interestingly when we measured the time each cell spent in division, the Par3 KO keratinocytes that divided correctly were significantly faster than the control counterparts when differentiation was induced. Our findings suggest that when Par3 is not present and cannot localize aPKC to the junctions, murine keratinocyte mitosis is altered at both temporal and functional levels in pro-differentiation context. We further tested possible causes for the aberrant divisions. We asked if this abnormal phenotype could be a consequence of centrosome amplification. Centrosomes are the major microtubules organizing centers (MTOCs) in mitotic and post-mitotic cells and are frequently associated with direct cell migration and apical polarity (Feldman & Priess 2012; Godinho et al. 2014; Sumiyoshi & Sugimoto 2012; Elric & Etienne-Manneville 2014). Centrosome amplification is common in aggressive tumors and currently accepted to have an important role in tumor progression. Surprisingly the loss of Par3 did not lead to centrosome amplification neither at undifferentiated nor at differentiated conditions. However centrosome amplification was clearly induced upon differentiation independent of the genotype, suggesting a differentiation-dependent mechanism. Remarkably the exact phenotype was observed when we performed the same experiments in spontaneously immortalized keratinocytes, supporting that loss of Par3 does not predispose to centrosome amplification.

We further asked if the incorrect divisions we have observed could fuel aneuploidy at short term (primary keratinocytes) and at long term (spontaneously immortalized keratinocytes). Cell aneuploidy is a chromosomic alteration in which the number of chromosomes of the aneuploid cell is different from the wild type

(Griffiths et al. 2000). It is a common feature in solid tumors and is driven by abnormal mitotic divisions as result of multipolar spindles (after incorrect cytokinesis or centrosome amplification), alterations in spindle attachment or by defective mitotic checkpoint response (Kops et al. 2005).

Similar to our findings concerning the centrosome numbers, Par3 deficiency did not predispose *per se* to aneuploidy, independently of the differentiation status. Indeed the differentiation onset disposed to cell aneuploidy irrespective of the genotype but highly correlated with centrosome amplification, either in primary or spontaneous immortalized cells. We did not find strong cumulative effect in the spontaneous immortalized keratinocytes that could suggest progressive phenotype aggravated by the *in vitro* culturing. Our results strongly suggest a differentiation-dependent effect that leads to centrosome amplification and cell aneuploidy. Interestingly in the skin context the presence of multinucleated cells is associated with skin inflammation. Multinuclear keratinocytes are reported in pathological conditions such as skin autoimmune pathologies, upon herpes infection or non-melanoma skin tumors (Tagami & Uehara 1981; Cohen et al. 2014). We speculate if somehow differentiation and perhaps Par3 deficiency sensitize keratinocytes through stress-signaling to onset of multinuclear cells and by that means explain in part our phenotypes.

The spindle apparatus is the main engine of the division by promoting the segregation of the chromosomes and by that means an equal distribution of the chromosomic content (Lara-Gonzalez et al. 2012). From our live cell imaging experiments, we had strong evidences of spindle alterations. In general the aberrant divisions observed in Par3 KO keratinocytes were “classical” tripolar divisions that have been well described (Keryer et al. 1984; Kalatova et al. 2015; Holland et al. 2012). Moreover, when analyzing centrosome numbers we focused mainly on the post-mitotic population independently of their microtubule nucleation capability. We then analyzed the spindle polarity, distinguishing between bipolars and multipolars spindle in mitotic primary cells. Interestingly, loss of Par3 induced spindle multipolarity at undifferentiated conditions when compared to the controls. In fact we could not find any spindle aberrancy in the control cells at LC. In contrast, when differentiation was induced spindle aberrancies were found in both control and Par3 KO cells and in a larger scale

when compared to the LC, suggesting that the contribution of loss of Par3 was less important once the differentiation was induced. Surprisingly all the spindles we analyzed were centrosome-associated and in fact, abnormal spindles correlated with centrosome amplification. For now we do not understand why loss of Par3 predisposes to the spindle aberrancies. On the one side it could be indirectly by affecting the centrosomes. Nevertheless we described here that we could not detect any causative link between Par3 deficiency and centrosome amplification but differentiation. Are the early differentiation features of Par3 already responsible for the centrosome amplification at LC? Why we did not detect that when we assessed the centrosomes? Are the centrosomes compromised functionally? In *C. elegans* loss of Par3 inhibits the reassignment of centrosomes at the apical surface in intestinal epithelia (Feldman & Priess 2012). On the other side Par3 and downstream targets could also impact the spindle directly or indirectly. Par3 associates with several microtubule-binding proteins such as dynein, kinesin/KIF3A, MARK2/Par1B (Schmoranzer et al. 2009; Nishimura et al. 2004; Lin et al. 2009). Additionally loss of Par3 in some cellular systems induces activation of the Tiam1-Rac pathway (Xue et al. 2012), which has a broad function in epithelial differentiation, motility and cell cycle, and it is involved in mitotic spindle formation (Whalley et al. 2015). We had observed differentiation-dependent phenotypes that predispose to centrosome amplification, aneuploidy and spindle aberrancies, suggesting a causative link between differentiation and the mitotic and post-mitotic aberrancies we had found. Parallel to these findings we observed Par3 deficiency dependent phenotypes in a mitotic context but at both differentiation conditions (aberrant divisions and shorter time in mitosis) in live cell imaging environment and at undifferentiated conditions (spindle aberrancies).

We further wanted to zoom out from the short period that it takes a cell to divide, and focused on overall cell cycle, where we expected to sieve out the penetrance of our observations apart from the mitosis context. We addressed this question using spontaneously immortalized keratinocytes at higher passages. Interestingly, Par3 deficient cells at LC exhibit a distinct profile from the controls that resembled the profile of control keratinocytes at differentiation conditions. When differentiation was induced both control and Par3 KO cells showed similar

profiles suggesting comparable cell cycle dynamics at HC. Our observations regarding the cell cycle suggested that loss of Par3 leads to premature differentiation even in absence of a differentiation stimulus. Interestingly these findings are supported by parallel observations from Iden Lab, that upon loss of Par3 several differentiation markers as Keratin-1, Loricrin, Involucrin and Filaggrin are upregulated (Ali et al, unpublished). Additionally despite the fact we have used spontaneously immortalized keratinocytes we did not observe considerable haploid or polyploid peaks that could indicate the presence of aneuploidy within the populations due to our described phenotypes.

Because the skin is the first barrier between an organism and its surrounding environment, it is continuously facing external aggressions. The UV radiation among others is a well described oncogenic factor and a primary cause of nonmelanoma skin cancer (El-Abaseri et al. 2005). Par3 deficient keratinocytes show a differential response to UV stress, mainly by overactivation of ATR and Chk1 at differentiated conditions (Letzian et al., unpublished). These proteins are important components of cell cycle checkpoints and are important players in DNA-damage response. These findings together with our above described observations prompted us to investigate the role of Par3 in mitosis also in the context of UV-mediated stress. We initially demonstrated that Par3 deficient keratinocytes divided faster than the controls at differentiation conditions. Surprisingly, upon UV irradiation this difference was even increased, suggesting a differential response to UV stress. For now we do not completely understand these differences and their relation with our molecular phenotypes. We speculate on one hand that a more active G2/M checkpoint can prevent a damaged cell to proceed in cell cycle but can on the other hand be an advantageous feature for damage coupling and apoptosis escaping. Nevertheless deeper functional and molecular analysis is required to unveil these questions.

Because the DNA damage response is strictly related to chromatin rearrangements we analyzed cell nuclei area as an indirect measure. We demonstrated here that in non UV context both Par3 KO and control nuclei were approximately of similar area, however, nuclei area at HC conditions were bigger than at LC. Interestingly upon UV irradiation Par3 deficient keratinocyte nuclei were significantly bigger than those of controls at undifferentiated conditions. Our

data supports a distinct nuclear organization in Par3 deficient keratinocytes upon UV stress at undifferentiated conditions that culminates in bigger nuclear area, by yet unknown molecular cause. For now we cannot explain this observation but we raise several hypotheses. A cell nucleus is highly organized (Deng & Blobel 2014). In fact nuclear organization is directly linked with gene expression. The modulation of chromatin folding and compactness has a direct impact over the genes accessibility. A cell that undergoes differentiation is shifting from one gene expression pattern to another and subsequently undergoing different chromatin rearrangements (Bhattacharya et al. 2009). If Par3 KO cells are prematurely differentiated, the differentiation program would require different subsets of genes that could result in a differential nuclear architecture. However this does not explain why we can see this difference exclusively upon UV-mediated stress. Heterochromatin reorganizes upon DNA damage. In fact chromatin-modifying factors localize after damage close to DNA breaks site (Oberdoerffer & Sinclair 2007). Par3 has previously been linked to DNA damage response upon Double Strand Break (DSBs) repair. Par3 itself was found to be a binding-partner of Ku70 and Ku80, subunits of DNA-dependent protein kinase (DNA-PK) an essential element of DSBs repair that has several phosphorylation sites in histones. In addition, Par3 KO cells have an impaired DSBs repair (Fang et al. 2007). We speculate that loss of Par3 could impact nuclear area by an impaired DNA-damage response and altered chromatin rearrangements.

The UVB irradiation is responsible for the majorities of radiation-induced skin lesions and is a major initiator of skin tumorigenesis (D'Orazio et al. 2013). At a molecular level, it affects the genomic integrity by provoking a range of lesions that constitute a repair challenge for the cell. Here we describe that upon UV-irradiation Par3 deficient keratinocytes had less 53BP1 positive foci, an important mediator of DNA strand break signaling and repair at undifferentiated conditions. We demonstrated that in a UV-mediated stress context loss of Par3 leads to a differential DNA damage response. We are intrigued by the fact the Par3 KO cells have less damage foci than the controls. However we are aware that this could result from a differential recovery or from different initial damage. Interestingly

when we compare Par3 KO cells at LC with control cells at HC it is quite striking that they behave in a similar fashion.

We know from our *in vivo* Ras-driven skin tumorigenesis experiments that Par3 deficiency predisposes to a rare type of cutaneous tumors in mice: the keratoacanthomas. Although being of increasing clinical relevance these are still poorly characterized tumors, and it is currently unclear which genetic and molecular defects cause keratoacanthoma. We analyzed cell divisions events in keratoacanthoma cross-sections and categorize them as asymmetrical and symmetrical. Surprisingly we have found several events that we identified as aberrant that we want to follow up further with careful quantification. We are intrigued by those events of aberrant divisions that are at basal layer, close to putative invasive sites. We speculate if this aberrant behavior is promoting invasiveness, or on the other hand, if the invading cells as consequence of losing their basal polarity cue (cell-substrate interactions at basal membrane) fail to orient their spindles.

In conclusion, in primary murine keratinocytes loss of Par3 predisposes to aberrant divisions when differentiation was induced. At HC Par3 KO keratinocytes that underwent a balanced division were significantly faster during their time in mitosis than the control cells, suggesting a faster mitotic progression. In addition, we demonstrated here that the onset of differentiation leads to centrosome amplification and aneuploidy *in vitro* independently of the genotype. Interestingly, at undifferentiated conditions we were able to detect spindle aberrancies in Par3 KO cells but not in the controls. Considering the cell cycle, Par3 deficient keratinocytes showed at LC a FACS profile comparable to control cells at differentiation, suggesting an early differentiation signature. From our UV studies we demonstrated here that Par3 deficient keratinocytes divide faster than controls upon UV-irradiation at HC. Additionally we measured the nuclei area and nuclei of Par3 KO cells were significantly bigger at undifferentiated conditions than those of control cells. We further analyzed the UV-mediated DNA damage response and found that Par3 deficiency led to differential damage response characterized by less 53BP1 foci at undifferentiated conditions. For now quite a few questions still need to be answered. Where does Par3 localize during mitosis? We have seen Par3 centrosomal staining in the past, and interactions

with microtubule-associating proteins are described. Does Par3 play a direct role in mitosis progression? Why does Par3 localize at centrosomes? Is it important for centrosome maturation or separation? On the other side there are several suggestions of an early differentiation phenotype. Nevertheless what does it mean? Additionally we have this dual role of Par3 in skin tumorigenesis with distinct types of tumors. Are the aberrant divisions contributing to the development of keratoacanthomas? Par3 has been shown to bind to Ku70/ Ku80 complex in the nucleus and to mediate damage repair, but does Par3 itself interact with DNA directly or indirectly? Par3 is a scaffold protein required to recruit other polarity proteins like aPKC to junctions. Does altered localization of Par3-interacting proteins do perhaps impact our phenotypes? What about Par3 isoforms like Par3B, which functions might be overlapping? Based on the present thesis and the resulting new questions, the design of follow-up studies is indicative. We will assess above questions by characterizing molecularly our models both *in vitro* and *in vivo*, and follow-up localization, interaction of Par3 and isoforms in skin homeostasis and tumorigenesis. For instance, we want to assess *in vitro* by live cell imaging the localization of GFP-bound Par3 and other members of Par complex during mitosis at both homeostasis and UV-mediated stress conditions. Depending on whether we identify Par3 to have a direct or indirect role in mitosis, we will access mechanistically the signaling pathways connecting Par3 to the mitotic regulation at both DNA and protein level. Mass spectrometry approaches currently performed in the Iden laboratory are expected to provide insight into potential Par3 binding partners and may unveil important signaling pathways in both the mitotic and protumorigenic context. In addition, we wish to characterize the aberrant divisions we observed *in vitro* by immunofluorescence of differentiation markers and cell cycle checkpoints markers. Moreover, we intend to characterize our tumors *in vivo* with respect to the aberrant divisions by immunofluorescence of markers for spindles together with basal layer and stroma, to unveil the nature of the aberrant divisions we observed. Additionally we want to assess the tumor dynamics *in vivo* by intra-vital imaging: mitotic capability and division, invasion and metastasis are just few of the features we plan to address.

We reveal a possible new function for Par3 in mitotic regulation and highlight the close relation between cell polarity and cell cycle in homeostasis and UV stress conditions. Interestingly we also observed that loss of Par3 predisposed to aberrant divisions, a common feature in tumor progression. Keratoacanthomas are fast growing tumors that arise commonly in sun-exposed skin however little is known about their etiology. Our findings contribute to understanding how loss of polarity impacts skin homeostasis and can contribute to keratoacanthomagenesis, a clinical relevant but poorly understood malignancy in humans.

MATERIALS & METHODS

Isolation of Primary Murine Keratinocytes

The primary keratinocytes were isolated from newborn K14Cre;Par3fl/fl;H2B-lokand dermal fractions were incubated overnight in dispase II solution. The next day the epidermis was detached from the dermis with forceps and incubated for 20 minutes in 0.5% TryPLE solution (Life Technologies) to dissociate keratinocytes. The epidermal cell suspension was pelleted and suspended in murine keratinocyte medium (DMEM/Ham´s F12, Biochrom AG) containing 10% calcium-depleted fetal calf serum.

Next to primary keratinocytes, also spontaneously immortalized and SV-40 immortalized keratinocytes were used. For the latter, SV40 viral particles were produced in Phoenix cells previously transfected with the plasmid pBabe-puro-SV40 according the Nolan Lab protocol (Stanford). Resulting supernatant was used to transduce passage one H2B-GFP^{+} primary keratinocytes.

Immunofluorescence Analysis: Monolayer Cultures

For monolayer cultures, primary or immortalized keratinocytes were seeded on Collagen I-coated 8 well LabTek Chamber Slides or 8 well SPL chamber slides, grown until confluency and then switched or not to high calcium (HC) levels (1,8mM). For immunofluorescence staining, monolayers were washed twice with PBS and fixed with PFA (4% PFA in PBS 10 min at room temperature (RT), followed by 15 min 0.5% Triton X-100 in PBS for permeabilization), ethanol/acetone (30 min 96% ethanol on ice, followed by 3 min incubation of ice-cold acetone at RT) or methanol fixation (10 min incubation at -20°C). Unspecific binding sites were blocked with 5% BSA/ PBS^{++} for 1 hr at RT, and monolayers subsequently incubated with primary antibodies diluted in AB buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.1% BSA) in a wet chamber overnight at 4°C. Incubation with AlexaFluor 488- and 594-conjugated secondary antibodies (Invitrogen) and DAPI as nuclei stain (Invitrogen) was carried out for 1 hour at RT in AB buffer in the dark. Immunostained cells on chamber slides were mounted in Mowiol or Dako mounting reagent. For the UV based experiments 24 hours prior to cell fixation, slides were irradiated with 5 J/cm^2 dose. Just before irradiation culture

medium was taken off and kept at RT. Cells were incubated in PBS during irradiation to avoid radiation absorption by the phenol-red present in the medium. After irradiation the former medium was added to the slides.

Immunohistochemistry

For immunohistochemistry, keratoacanthomas from Par3eKO mice were fixed with 4% PFA and embedded in paraffin. Paraffin sections (4 µm) were deparaffinized and the antigens retrieved with Dako buffer pH6, followed by blocking with 10% donkey serum in PBS for 1 hour at RT. Slides were incubated with primary antibodies followed by a washing step and incubation with AlexaFluor 488 and 594-conjugated secondary antibodies (Invitrogen) diluted in AB buffer for 1hour at RT. Nuclei were counterstained with DAPI (Invitrogen) and the slides were mounted in Mowiol mounting reagent.

Live Cell Imaging and Image Analysis

Primary keratinocytes isolated from H2B-GFP^{+} mice were seeded on Collagen I-coated 8 well µ-Slides (IBIDI®) grown until confluency and then switched or not to HC levels. Approximately 4,8x10^{4} and 6,0x10^{6} cells per well for LC or HC conditions, respectively, were seeded cells were meant for LC or HC incubation. The time-lapse microscopy was performed using a Leica® DMI 6000 equipped with Pecon® PM2000 incubator. The cells were maintained at 37°C, 5% CO_2, and time lapse images were captured every 10 min during a period of 12-24 hours. Subsequent analysis of the material was performed with Leica X software and Image J.

Live Cell Imaging upon UV Irradiation

For Live Cell Imaging upon UV irradiation the same protocol from our other live imaging experiments was followed, but 24 hours after CS the culture medium was removed and kept at RT. The cells were sustained in PBS and irradiated with a dose of 5 J/cm^{2}. After irradiation the culture medium was added again to the wells. After 12 hours we started the imaging protocol. Post-acquisition image analysis was performed with Leica X software and Image J.

Cell Synchronization

Immortalized cultures were serum-starved for 36 hours to achieve G1 phase cell cycle arrest with keratinocyte medium without FCS and defined signaling components. After that period cells were released with complete keratinocyte medium containing FCS.

FACS DNA content analysis

For determination of cell cycle progression flow cytometric DNA quantitation was performed as described elsewhere (Günschmann et al. 2013). Cells were stained with propidium iodide to stain chromosome content, and for subsequent FACS analysis a BD LSRfortressa® was used.

Typically, 50.000 cells were examined in each experiment. Gating was performed to exclude fluorescence background signals (unstained control cells), and debris and cell aggregates were excluded based on forward and sideward scatter characteristics. Subsequent analysis and model assumption was performed with ModFit 4.0 (Verity Software).

Nuclei Area Measurements using Cell Profiler

Live Cell Imaging data were analysed using the open-source software Cell Profiler (Broad Institute, Cambridge, MA). The following pipeline was applied: Conversion of the raw .tiff data from color to greyscale image, followed by *Identify Primary Objects* module to select nuclei based on the fluorescence signal as objects to be evaluated. Applying the *Measure Size and Shape* module yielded various morphological parameters such as nucleus area. Quantitated data were exported in .csl format using the *export to spreadsheet* module. Approximately 400 cells were evaluated per experiment.

Statistical Methods

Two-tailed unpaired student's t tests were performed to assess for statistical significance. Corresponding p-values are indicated in the figures (asterisks) and detailed in figure legends. Data represent mean of at least three independent experiments and error bars indicate standard deviation (±SD). For the quantification of mitosis duration, a non-linear mixed effects model (nlme, CRAN & R Foundation) was used. Following this approach we take into consideration

the inter-replicates variation, in this case due to cell-cell variability present in each experiment and different sample size.

Antibodies

During this study the following antibodies were used: mouse monoclonal against ɣ-Tubulin (SIGMA-Aldrich, no T6557) dilution 1:1000, α-tubulin (SIGMA-Aldrich, no T6074) dilution 1:1000, rabbit polyclonal against pericentrin (BioLegend, no PRB-432C) dilution 1:300, 53BP1 (abcam, no ab 36823) dilution 1:500. For secondary detection, donkey antibodies against mouse and rabbit IgG conjugated with AlexaFluor fluorophores (Invitrogen) were used dilution 1:500.

SUPPLEMENTARY DATA

Movies (1-4): primary keratinocytes

M1: control LC

M2: Par3 KO LC

M3: Control HC

M4 Par3 KO HC

Movies (5-8): SV40 immortalized keratinocytes

M5: Control LC

M6: Par3 KO LC

M7 Control HC

M8: Par3 KO HC

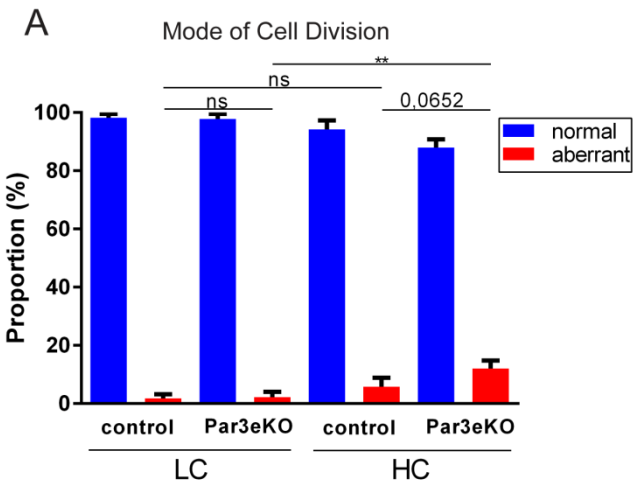

Figure S1. Aberrant Divisions in Control and Par3 KO SV40 Keratinocytes

(A) Aberrant divisions quantification (n=3), bars represent mean+SD; ns: $p > 0.05$; **: $p \leq 0.01$.

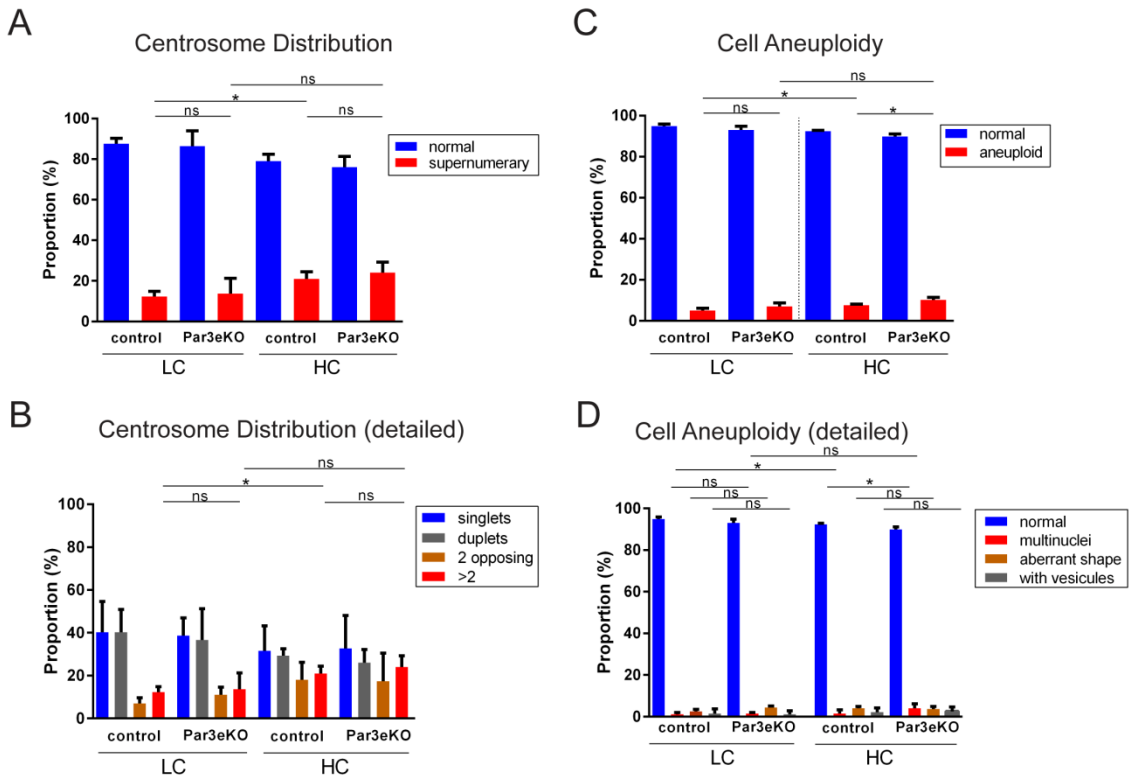

Figure S2. Centrosome Amplification and Nuclear Morphology in Control and Par3 KO Spontaneously Immortalized Keratinocytes

(A) Quantification of number of cells with normal and supernumerary centrosomes (n=3), bars represent mean±SD; ns: $p > 0.05$; **: $p \leq 0.01$.
(B) Overall centrosome distribution (n=3), bars represent mean±SD; ns: $p > 0.05$; *: $p \leq 0.05$.
(C) Quantification of cell aneuploidy (n=3), bars represent mean±SD; ns: $p > 0.05$; *: $p \leq 0.05$.
(D) Quantification of nuclear phenotypes (n=3), bars represent mean±SD; ns: $p > 0.05$; *: $p \leq 0.05$.

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