

Journal Pre-proof

Embryonic NIPP1 depletion in keratinocytes triggers a cell-cycle arrest and premature senescence in adult mice

Marloes C.M. Jonkhout, Tijs Vanhessche, Mónica Ferreira, Iris Verbinnen, Fabienne Withof, Gerd Van der Hoeven, Kathelijne Szekér, Zahra Azhir, Wen-Hui Lien, Aleyde Van Eynde, Mathieu Bollen

PII: S0022-202X(24)00167-2

DOI: <https://doi.org/10.1016/j.jid.2024.02.009>

Reference: JID 4183

To appear in: *The Journal of Investigative Dermatology*

Received Date: 28 July 2023

Revised Date: 24 January 2024

Accepted Date: 9 February 2024

Please cite this article as: Jonkhout MCM, Vanhessche T, Ferreira M, Verbinnen I, Withof F, Van der Hoeven G, Szekér K, Azhir Z, Lien W-H, Van Eynde A, Bollen M, Embryonic NIPP1 depletion in keratinocytes triggers a cell-cycle arrest and premature senescence in adult mice, *The Journal of Investigative Dermatology* (2024), doi: <https://doi.org/10.1016/j.jid.2024.02.009>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2024 The Authors. Published by Elsevier, Inc. on behalf of the Society for Investigative Dermatology.



Embryonic NIPP1 depletion in keratinocytes triggers a cell-cycle arrest and premature senescence in adult mice

Marloes C. M. Jonkhout¹ (0000-0002-6099-927X), Tijs Vanhessche^{1*} (0000-0003-4196-6357), Mónica Ferreira^{1*} (0000-0001-9609-5099), Iris Verbinnen¹ (0000-0002-3681-1628), Fabienne Withof¹, Gerd Van der Hoeven¹, Kathelijne Szekér¹, Zahra Azhir¹ (0000-0003-0476-1547), Wen-Hui Lien² (0000-0002-2282-3169), Aleyde Van Eynde¹ (0000-0002-3462-4976) and Mathieu Bollen¹ (0000-0003-4975-1510)

¹Laboratory of Biosignaling & Therapeutics, KU Leuven Department of Cellular and Molecular Medicine, University of Leuven, Belgium

²de Duve Institute, Faculty of Medicine, Université catholique de Louvain, Brussels, Belgium

*contributed equally

Corresponding author:

Aleyde Van Eynde
KU Leuven, Campus Gasthuisberg,
Herestraat 49, O&N1, PB901,
B-3000 Leuven, Belgium.
Phone: +32-16-33 06 44
E-mail: Aleyde.VanEynde@kuleuven.be

Keywords: quiescence, senescence, NIPP1, SASP, p21, p16/Ink4a, p19/Arf

Short title: NIPP1 deletion leads to senescence

Abbreviations: DDR, DNA-damage response; DP, dermal papilla; HF, hair follicle; HFSC, hair follicle stem cells, NIPP1, nuclear inhibitor of PP1; PK, primary keratinocytes; PP1, protein phosphatase 1; SASP, senescence associated secretory phenotype;

Journal Pre-proof

ABSTRACT

NIPP1 is a ubiquitously expressed regulatory subunit of protein phosphatase-1. Its embryonic deletion in keratinocytes causes chronic sterile skin inflammation, epidermal hyperproliferation and resistance to mutagens in adult mice. To explore the primary effects of NIPP1 deletion, we first examined hair-cycle progression of NIPP1 skin knockouts (SKOs). The entry of the 1st hair cycle in the SKOs was delayed due to prolonged quiescence of hair-follicle stem cells (HFSCs). In contrast, the entry of the 2nd hair cycle in the SKOs was advanced as a result of precocious activation of HFSCs. The epidermis of SKOs progressively accumulated senescent cells and this cell-fate switch was accelerated by DNA damage. Primary keratinocytes from SKO neonates and human NIPP1-depleted HaCaT keratinocytes failed to proliferate and showed an increase in the expression of cell-cycle inhibitors (p21, p16/Ink4a and/or p19/Arf) and senescence-associated-secretory-phenotype (SASP) factors as well as in DNA damage (γ H2AX and 53bp1). Our data demonstrate that the primary effect of NIPP1 deletion in keratinocytes is a cell-cycle arrest and premature senescence that gradually progresses to chronic senescence and likely contributes to the decreased sensitivity of SKOs to mutagens.

INTRODUCTION

NIPP1 (nuclear inhibitor of protein phosphatase-1), also known as PPP1R8, is a regulator of the nuclear pool of PP1 (Heroes et al. 2013; Minnebo et al. 2013; O'Connell et al. 2012). The PP1:NIPP1 holoenzyme is inactive under basal circumstances, but becomes active in conditions of DNA damage through phosphorylation of NIPP1 at Y335 by (a) Src-family kinase(s) (Wu et al., 2023). NIPP1-Y335 phosphorylation results in the de-inhibition of associated PP1 and an enhanced substrate recruitment via the ForkHead-Associated (FHA) domain of NIPP1, resulting in substrate dephosphorylation. All known NIPP1 FHA-ligands (CDC5L, EZH2, MELK, SF3B1, KAP1) play a role in DNA-damage repair (Beke et al. 2015; Rondinelli et al.

2017; Savage et al. 2014; Smith-Moore et al. 2018; Zhang et al. 2009), but it remains unclear how their dephosphorylation by PP1:NIPP1 affects DNA repair. Since PP1:NIPP1 activation is a late response to DNA damage, it likely contributes to the timely recovery from DNA-damage repair (Wu et al. 2023). Consistent with this view, the deletion of NIPP1 from liver or skin results in constitutive DNA-damage repair, as evidenced by an increase in the expression of DNA-repair factors and a strong reduction in the sensitivity to chemical mutagens (Verbinnen et al. 2020a). Conversely, the overexpression of a PP1-NIPP1 fusion protein or the constitutive activation of PP1:NIPP1 by expression of a phospho-mimicking NIPP1 mutant (Y335E) causes the accumulation of DNA damage, consistent with precocious DNA-damage repair silencing (Winkler et al. 2018; Wu et al. 2023).

In addition to its role in DNA-damage repair, NIPP1 is also (in)directly implicated in cell proliferation (Ferreira et al., 2018a). NIPP1 depletion reduces the proliferation rate of embryonic stem cells (Van Eynde et al. 2004), spermatogonia (Ferreira et al. 2017), and cultured cancer cells (Van Dessel et al. 2015). In contrast, the embryonic liver- or skin-specific knockout (SKO) of NIPP1 results in enhanced proliferation in adult mice of biliary epithelial cells and keratinocytes, respectively (Boens et al. 2016; Verbinnen et al. 2020b). Skin hyperplasia in the SKOs is associated with a massive expression of pro-inflammatory chemokines by keratinocytes, culminating in sterile skin inflammation (Verbinnen et al., 2020b). The hyperplastic phenotype of SKO keratinocytes is suppressed by inhibition of inflammation, indicating that hyperproliferation is a secondary effect caused by immune-cell infiltration. The molecular mechanism of the increased expression of chemokines and DNA-repair factors after NIPP1 depletion in keratinocytes remains unknown.

To differentiate between primary and secondary effects of NIPP1 depletion, and to gain further insights into the role of NIPP1 in cell proliferation, we compared the phenotype of SKOs before and after the development of inflammation. We focussed on the first and second

postnatal hair cycles in back skin, which are characterized by synchronized, consecutive phases of growth (anagen), regression (catagen) and quiescence (telogen), and are critically dependent on the cyclic proliferation of hair follicle stem cells (HFSCs) (Alonso and Fuchs 2006; Müller-Röver et al. 2001; Schneider et al. 2009). We found that HFSCs in the 1st telogen of SKO hair follicles (HFs) displayed prolonged quiescence. In contrast, HFSCs in the 2nd telogen were precociously activated. Proliferating keratinocytes in aging SKOs became senescent and this altered cell fate quickened by the induction of DNA damage. A senescent phenotype was also observed in primary keratinocytes from newborn SKO mice and in human NIPP1-depleted HaCaT cells. Our data suggest that the primary effect of NIPP1 depletion in keratinocytes is a cell-cycle arrest that gradually develops into chronic senescence.

RESULTS

NIPP1 depletion affects the hair cycle in an age-dependent manner

To delineate the contribution of NIPP1 to hair growth in mouse back skin, we used the previously generated NIPP1 skin-knockout model (Verbinnen et al., 2020b). In this model, NIPP1 is selectively removed from keratinocytes starting from embryonic (E) day 14.5 through recombination of a floxed *Ppp1r8* allele by Cre-recombinase under control of the *Keratin-14* promoter. Mice with the genotypes *Tg(Krt14-Cre)/Ppp1r8^{fl/+}* and *Tg(Krt14-Cre)/Ppp1r8^{fl/-}* were used as control (CTR) and skin-knockout (SKO) mice, respectively. We confirmed the depletion of NIPP1 in the back skin using reverse transcription-quantitative PCR (RT-qPCR) (Figure S1a) and immunostaining (Figure S1b). As expected, NIPP1 in the SKOs was depleted in the epidermal layer and the contiguous epidermal components of dorsal HFs, including the bulge and hair germ (HG), but not in the dermis and dermal papilla (DP) (Figure S1b). To assess the first and the second telogen-anagen transition, we monitored hair re-growth after shaving at either postnatal day (PD) 19-21 (telogen of the 1st hair cycle; further denoted as PD20) or PD31

(anagen of the 1st hair cycle), respectively (Figure 1a) (Kimura-Ueki et al. 2012; Li et al. 2020). Hair-cycle progression was followed by the transition from pink (telogen) to grey/black (mid anagen), and hair regrowth (late anagen) (Müller-Röver et al. 2001; Stenn and Paus 2001). Following shaving at PD20, both CTR and SKO HF^s were in the 1st telogen (telogen-1; Figure 1b, c). However, while CTR HF^s recovered the hair coat at PD31, the SKO HF^s showed a delay in hair coat recovery of 5.5 ± 1.5 ($n = 5-6$) days, hinting at a prolonged telogen-1 in SKOs (Figure 1d). After CTR and SKO HF^s entered the 2nd telogen at ~PD43, as demonstrated by pink skin color (Figure 1e-f), CTR HF^s remained in telogen-2 until ~PD115 whereas SKO HF^s entered the 2nd hair cycle precociously (Figure 1g) and formed the full hair coat at ~PD84 (versus ~PD119 in CTR; Figure 1h). These differences were gender-independent (Figure S1c, d) and indicative for precocious HFSC activation and hair-cycle entry in the SKOs. We also performed back-skin depilation experiments of telogen HF^s at PD21 to induce a synchronized anagen initiation and HFSC activation (Müller-Röver et al. 2001). These experiments disclosed a delayed depilation-induced anagen entry in the SKOs (Figure 1i and S1e, f), consistent with a prolonged telogen-1 (Figure 1d). Finally, a morphological analysis of hairs plucked at PD56 revealed that the main hair types were formed in similar proportions in CTRs and SKOs, i.e. ~2% guard, ~20% awl and ~78% zigzag hairs (Duverger and Morasso 2009) (Figure 1j). However, the awl and zigzag hairs, but not the guard hairs, were significantly shorter in the SKOs (Figure 1k), suggesting a shorter growth phase (anagen 1) of the 1st hair cycle, without perturbation of the entry of telogen-2 (Figure 1l) (Plikus 2012). Nevertheless, we cannot exclude that the shorter hair length of the SKOs is (partially) attributed to a slower hair-growth rate. We also noted that ~70% of zigzag hairs, characterized by 1 medulla column and a zigzag bending, lost their bending in SKOs (Figure S1g, h), suggesting a (minor) defect in hair differentiation. In summary, the embryonic deletion of NIPP1 in epidermal cells resulted in a prolonged telogen

of the 1st hair cycle and a precocious entry of the 2nd hair cycle (Figure 1l), with only minor effects on hair differentiation.

Hair-follicle stem cells in the 1st telogen of SKO HF show a delay in activation

Based on our macroscopic data (Figure 1), we selected PD21, PD28, PD56 and PD77 for further histological analysis of the dorsal skin. Haematoxylin & Eosin (H&E) stainings of CTR and SKO skin showed that CTR and SKO HF at these ages all resided in the dermal layer, except for SKO HF at PD77, which extended deep into the subcutis, indicating that they were in late anagen (Figure 2a). Accordingly, morphological classification of dorsal HF (see supplemental Materials & Methods section for experimental details), demonstrated that most HF of CTRs and SKOs were in telogen-1 at PD21, telogen-1 and early anagen-1 at PD28 and telogen-2 at PD56 (Figure 2b). However, at PD77 nearly all CTR HF resided in telogen-2, while the HF of SKOs had already progressed to mid/late anagen-2, consistent with their permeation into the subcutis (Figure 2a).

To determine whether the altered hair-cycle entries in SKOs (Figure 1) were due to differences in HFSC activation, we performed immunostainings on dorsal skin for both CD34, a bulge HFSC marker, and Ki67, a proliferation marker. Ki67⁺/CD34⁺ cells were denoted as ‘proliferating bulge HFSCs’ (Figure 2c, d), and Ki67⁺/CD34⁻ cells between the bulge and dermal papilla were designated as ‘proliferating HG cells’ (Figure 2e, f). At PD21, there was no difference in the number of proliferating bulge HFSCs (Figure 2d), but the SKOs had less proliferating HG cells (Figure 2f), indicating that SKO HF were more dormant than their CTR counterparts, in accordance with the delayed 1st telogen-anagen transition in the SKOs (Figure 1b-d). However, HF from SKOs did eventually activate, as demonstrated by the increased number of proliferating HFSCs and HG cells at PD28 (Figure S2a, b). At PD28, the proliferation rate of SKO HG cells was even higher than that of CTR cells (Figure 2f), pointing

at a delayed HG activation of SKO HFSCs. In contrast, at PD56 and/or PD77, the number of proliferating HFSCs and HG cells in the SKOs was significantly higher than in CTRs (Figure 2d), in agreement with the precocious 2nd telogen-anagen transition in the SKOs (Figure 1e-h). Premature HFSC activation in SKOs at PD56 did not appear to be caused by a loss of the old bulge, as shown by CD34 immunostainings (Figure 2c). Neither did a flow-cytometry analysis reveal differences in the number of basal (CD34^{high}/ITGA6^{high}) and suprabasal (CD34^{high}/ITGA6^{low}) HFSCs between CTRs and SKOs at PD56 (Figure S2c, d). Also, the number of FOXP3⁺ regulatory T (T_{reg}) cells, which have also been implicated in HFSC activation (Ali et al. 2017), was not different between CTRs and SKOs at PD56/77 (Figure S2e, f). However, we observed a significant increase in CD45⁺ immune cells in SKO back skin at PD56, as reported earlier (Verbinnen et al. 2020b), but not yet at PD21 and PD28 (Figure 2g, S2g). These data show that the premature onset of the 2nd anagen in SKOs coincides with increased immune-cell infiltration, indicating that the precocious activation of HFSCs at anagen of the 2nd hair cycle could be a secondary effect caused by an immune response. In conclusion, HFSCs primarily showed prolonged quiescence and delayed activation when NIPP1 was depleted.

Primary keratinocytes from newborn SKOs undergo premature senescence

To identify cell-autonomous effects of the embryonic deletion of NIPP1 in skin, we isolated primary keratinocytes (PKs) from the dorsal skin of newborn CTR and SKO pups. The removal of NIPP1 in the SKO PKs was verified by RT-qPCR (Figure S3a). PKs from SKOs were severely impaired in both proliferation (Figure 3a) and colony formation (Figure 3b, c). This phenotype did not seem to result from an adhesion defect, as the confluency of the seeded SKO PKs did not decrease over time (Figure 3a). Moreover, PKs of SKOs harvested immediately (0 days) or 2 days after seeding on collagen-I/fibronectin-I coated plates, showed a normal

expression of the adhesion marker *Itga6* (Figure 3d). RT-qPCR analysis also revealed a similar decrease of differentiated CTR and SKO PKs after a 2-day seeding, as evidenced by the same loss of differentiation markers (*Krt10* and *Lor*) (Figure 3e and S3b). However, the *Krt14* transcript levels were not affected within the same period (Figure S3c).

A flow-cytometric analysis showed that, 2 days after seeding, $58 \pm 8 \%$ ($n = 4$) of SKO PKs were in the G₀/G₁ phase, as compared to only $42 \pm 8 \%$ ($n = 4$) of CTR PKs (Fig. 3f, g). The CTR and SKO cells showed a minor difference in the expression of the early differentiation markers *Invol*, *Krt1* and *Krt10* (Fig. S3d-f), but not in that of the late differentiation markers *Krt16* and *Krt17* (Fig. S3g-h) at days 2-6 post-seeding. However, the higher proportion of SKO PKs in G₀/G₁ and the proliferation arrest correlated with an increased expression of the cell-cycle inhibitor p21, encoded by *Cdkn1a*, as shown by both RT-qPCR (Figure S3i) and immunostainings (Figure 3h, i). At days 4-6 post-seeding, we also detected an increased expression of the cell-cycle inhibitor *p19/Arf*, encoded by the *Cdkn2a* locus (Figure 3j). However, the expression of *p16/Ink4a*, encoded by the same locus, was not affected (Figure S3j).

In addition to a p19/21-mediated cell-cycle arrest, we obtained various lines of evidence for the development of premature senescence in SKO PKs. First, immunostainings revealed a decreased level of the nuclear intermediate filament LaminB1 and the nuclear chromatin protein HMGB1 as well as an increased level of the DNA-damage sensor 53bp1 in SKO PKs at 6 days after seeding (Figure 4a-f). Second, a persistent DNA damage was detected in SKO PKs, as indicated by an increased level of γ H2AX at 2-6 days post seeding, as shown by immunostaining (Figure 4g, h). Finally, various proteins of the ‘senescence-associated secretory phenotype’ (SASP) were upregulated in SKO PKs, including the chemokines *Cxcl1* and *Ccl2* (Figure 4i and S4a), the interleukins *Il1a* and *Il1b* (Figure 4j and S4b) and the matrix metalloproteinases *Mmp3* and *Mmp9* (Figure 4k and S4c). However, the SASP factor *Il6* was

not increased (Figure S4d). This is consistent with the well-established heterogeneity of SASP composition as a result of differences in the cellular context and timing. Taken together, our data demonstrate that NIPP1-depleted PKs suffered from DNA-damage induced premature senescence since DNA damage was already increased from 2 days after seeding.

Keratinocytes in SKOs also gradually develop senescence

We noted that 17% of SKOs (n=30) died within 480 days after birth, as compared to only 3% for the CTRs (n=30). This suggested that SKOs aged faster, consistent with the increased accumulation of senescent cells in SKO skin. The pelage of 63-weeks-old SKOs could also be visually distinguished from CTR mice by sparser hair distribution (global alopecia), a hair coat with a more greasy and prickly appearance, and a depigmented and flaky tail (Figure S5a). This prompted us to investigate whether the expression levels of various senescence markers, including cell-cycle inhibitors, SASP factors, γ H2AX, Senescence-Associated (SA)- β -galactosidase, LaminB1 and HMGB1, were also affected in the skin of NIPP1 SKOs. In tail skin, the *Cdkn2a* locus was hyper-activated in the SKOs from 4 weeks (p19/Arf) and 8 weeks (p16/Ink4a) onwards, as detected by RT-qPCR (Figure 5a, Table S1), RNA-Seq (Table S1 in Verbinen et al., 2020b) and immunoblotting (Figure 5b). We also explored the expression of the *Cdkn2a* locus in basal epidermal cells (BECs, CD34^{low}/ITGA6^{high}) and HFSCs (CD34^{high}/ITGA6^{high}) that were purified from tail skin of 8-weeks-old CTR and SKO mice by fluorescence-activated cell sorter (FACS) (Figure S5b). The relative expression of the *Cdkn2a* locus was increased in both SKO HFSCs and BECs, suggesting that the *Cdkn2a* locus is activated in various epidermal cells and is not limited to a specific cell type (Figure 5c). In contrast, the expression of p21 was unaltered, except at 4 weeks, in the SKO tail skin (Figure 5a, Table S1). Various SASP factors (*Ccl1*, *Ccl2*, *Cxcl1*, *Il1b* and *Mmp9*), but not *Il6*, were also strongly upregulated in the SKO tail skin from 3 and/or 4 weeks onwards (Figure 5a, S5c and

Table S1). The number of γ H2AX-positive cells was >2-fold higher than in CTRs (Figure 5d), which is in agreement with the phenotype in SKO PKs (Figure 4g, h). The development of the (premature) senescent phenotype in tail skin at 8 weeks was also confirmed by the increased number of cells that stained positive for SA- β -galactosidase, as quantified by flow cytometry (Figure 5e and S5d). Importantly, the increased expression of cell-cycle inhibitors and (some) SASP factors was still detected at 40 weeks (Figure 5a, Table S1), hinting at chronic senescence. Consistent with this interpretation, the level of the nuclear intermediate filament LaminB1 (*Lmnb1*) was decreased in SKO tail skin at 40 weeks, as shown by RT-qPCR and immunostaining (Figure 5a and 5f, Table S1) (Freund et al. 2012). Together, these data demonstrate that (a subset of) keratinocytes in the tail of SKOs gradually developed chronic senescence and accumulated DNA double-strand breaks. The phenotype in the back skin of SKOs turned out to be much milder and was limited to an increased expression of SASP factors from 3 weeks onwards (Figure 5g, Table S2). The global levels of p16/Ink4Aa, p19/Arf and p21 transcripts were not altered at 52 weeks in the SKO back skin (Figure 5g, Table S2). However, immunostaining showed a decreased expression of LaminB1 (Figure 5h) as well as HMGB1 (Figure 5i) in back skin of 8-week-old SKOs. Furthermore, RT-qPCR analysis revealed a >2-fold increased expression of p21 in basal HFSCs (CD34^{high}/ITGA6^{high}) and a 39%-increased expression in basal epidermal cells (BECs, CD34⁻/ITGA6^{high}) that were isolated from the back skin of SKOs of 3 weeks (Figure 5j and S5e). Both HFSCs and BECs were positive for the expression of *Krt14*, while the stem cell marker *Cd34* was only expressed in HFSCs, confirming the identity of the isolated cell population (Figure S5f, g). In conclusion, our data disclosed senescence in SKO skin, which was first detected in cultured PKs, and developed faster in tail skin than in back skin.

We speculated that the distinct susceptibility of dorsal and tail skin to the development of senescence was linked to structural differences and/or to the higher exposure of tail skin to

environmental and physical stress (Quigley et al. 2016). To examine whether enhanced stress signaling in tail skin promotes the development of senescence, we investigated the effect of a topical treatment with a DNA-damaging agent on the onset of senescence in the back skin of SKOs. More specifically, we examined the expression of cell-cycle inhibitors and SASP factors after a short-term (4h) application of the chemical mutagen 7,12-dimethylbenz[a]anthracene (DMBA) for 7-8 weeks. This treatment indeed induced a manifold increase in the expression of p16/Ink4a and p19/Arf, but not p21, in SKO back skin, and these effects were absent (p16/Ink4a) or less pronounced (p19/Arf) in CTR back skin (Figure 5k-m). The number of DMBA-induced γ H2AX-positive cells was also about twofold higher in treated SKOs compared to CTRs (Verbinnen et al. 2020a). Importantly, *Ccl1* and *Ccl2* expression was already increased in the SKOs before application of DMBA (Figure 5n), indicating that the induction of senescence was not merely the consequence of a SASP response, but was critically dependent on the accumulation of DNA damage after DMBA treatment.

Depletion of NIPP1 in human keratinocytes also causes senescence

Finally, we examined whether the senescent phenotype of NIPP1-depleted cells was a peculiarity of mouse keratinocytes or also applied to human keratinocytes. We have previously reported that the siRNA-mediated knockdown of NIPP1 in human HaCaT cells reduced cell proliferation and induced expression of various cytokines, in agreement with a SASP response (Verbinnen et al. 2020b), and similar to what we observed for mouse PKs (Figure 3 and 4). Consistent with the composition of the SASP response in SKO PKs and SKO mice, we found that *IL1A*, but not *IL6*, was increased after siRNA-mediated knockdown of NIPP1 in HaCaT cells (Figure 6a and S6). Human *IL8*, which does not exist in mice, but is considered as the functional homolog of mouse *Cxcl1* and *Cxcl2*, was also increased in NIPP1-depleted HaCaT cells (Figure 6b). Furthermore, we found that NIPP1 depletion also resulted in an increase in

transcription levels of p21 from 24h onwards and of p16/INK4A at 72h, but had no effect on the expression of p14/ARF, the human p19/Arf homologue (Figure 6c-e). A consecutive expression of p21 and p16/INK4A has also been reported in a senescence model induced by EZH2-depletion (Ito et al. 2018). Immunoblotting confirmed an increased expression of p21 protein at 48 and 72h after siRNA-mediated knockdown of NIPP1 in HaCaT cells (Figure 6f, g). However, a siRNA-mediated knockdown of p53 eliminated the increased expression of p21 in NIPP1-depleted cells, suggesting that the p21 upregulation depends on p53 (Figure 6h). NIPP1-depleted cells also showed a decrease in the expression of LaminB1 (Figure 6i) and an increase in the signal of γ H2AX (Figure 6j, k), underscoring the senescent phenotype. Together, our data demonstrate that the senescent phenotype induced by NIPP1 depletion is conserved between human and mouse keratinocytes.

DISCUSSION

To delineate the primary effects of NIPP1 depletion in keratinocytes, we first compared hair-growth phenotypes of SKOs during adolescence and adulthood. The 2nd hair cycle in SKOs was advanced, which can be explained by hyperproliferation of HFSCs caused by SASP-induced immune-cell infiltration (Figure 1, 2). In contrast, the entry into the 1st hair cycle, before the onset of an inflammatory response, was delayed in the SKOs and correlated with prolonged quiescence of HFSCs. Quiescence is a reversible cell-cycle arrest that prevents stem-cell depletion and reduces cell-fate plasticity (Lee et al. 2021). Exit from quiescence and the concomitant activation of HFSCs is regulated by cell-cycle regulators like p21 that hamper the G₁/S-transition through inhibition of cyclin-dependent kinases (CDK) that phosphorylate the Retinoblastoma protein (Blanpain et al. 2004; Engeland 2022; Kulaberoglu et al. 2016; Lee et al. 2013). Consistent with prolonged HFSC quiescence, HFSCs purified from SKO HFs at telogen-1 expressed more p21 than CTR HFSCs (Figure 5). However, a p21-associated cell-

cycle arrest was also apparent in cultured primary keratinocytes from SKO neonates (Figure 3), demonstrating that more differentiated proliferating keratinocytes were also affected. The epidermis of adolescent SKO mice also expressed SASP factors, but did not yet show an increased immune-cell recruitment, hinting at an early phase of senescence (Figure 5). Eventually, this phenotype developed into late and chronic senescence in young adult SKOs, characterized by a strongly increased expression of cell-cycle inhibitors (p16/Ink4a and/or p19/Arf) and SASP factors, and the decreased expression of LaminB1 (Figure 5). Secreted SASP factors likely contributed to the expansion and maturation of the senescent phenotype in adult SKOs, as they are known to induce senescence in neighbouring cells (Wang et al. 2022) and to recruit immune cells, culminating in inflammation (Herranz and Gil 2018). Intriguingly, senescence developed faster in tail epidermis than in back epidermis (Figure 5). This may be due to more pronounced DNA damage in tail skin caused by enhanced exposure to physical stress (Quigley et al. 2016), resulting in a persistent DNA-damage response (DDR) (Hernandez-Segura et al. 2018). Consistent with enhanced DDR signaling, PKs and adult SKO epidermis indeed showed augmented levels of γ H2AX (Figure 4 and 5).

NIPP1 belongs to the large group of Regulatory Interactors of Protein Phosphatase One (RIPPOs) that control the activity and substrate specificity of the associated PP1. Previous data linked PP1 to senescence through association with a RIPPO, known as Rap1-interacting factor 1 or RIF1 (Zhang et al. 2023). Here, we identified NIPP1 as an extra player in the development of senescence. While loss of NIPP1 induces senescence (this work), knockdown of RIF1 or PP1 reduced premature senescence (Zhang et al. 2023). We speculate that this different response is linked to the different substrate selectivity of the holoenzymes PP1:RIF1 and PP1:NIPP1. In addition to the well-established role of reversible protein (de)phosphorylation in the control of senescence, other post-translational modifications, such as (de)acetylation, are also involved. SIRT7 is a member of the sirtuin (SIRT) family of lysine deacetylases that exhibits deacetylase

activity towards histone H3K18 as well as non-histone substrates (Raza et al. 2024). Overexpression of SIRT7 delays stress-induced premature senescence in cells treated with doxorubicin (Blank and Grummt 2017; Kiran et al. 2015) and SIRT7 levels decline upon aging and in senescent cells (Raza et al. 2024). Intriguingly, loss of SIRT7 in mice delays hair-follicle cycling and hair growth by hindering telogen-to-anagen transition, and also correlates with premature aging (Li et al. 2020; Raza et al. 2024), similar to what we observed after NIPP1 depletion.

Our data suggest that the increased expression of p21 is due to a p53-mediated transactivation of the p21-encoding *Cdkn1a* gene (Figure 6) (Engeland 2022; Fischer 2017; Fischer et al. 2014). However, this does not exclude the involvement of transcriptional (co-) repressors such as RUNX1 or EZH2. RUNX1 represses *CDKNA1* expression through recruitment of the Polycomb Repressive Complex 1 (PRC1), while EZH2 inhibits *CDKNA1* expression through recruitment of histone deacetylase 1 (HDAC1) and/or trimethylation of histone H3 at Lys 27 (Adhikari and Davie 2020; Fan et al. 2011; Lee et al. 2013; Yu et al. 2012). As the deletion of NIPP1 in spermatogonia resulted in the hyperphosphorylation and proteasomal degradation of EZH2 (Ferreira et al. 2018b), it is tempting to speculate that the hypoproliferation of NIPP1-depleted keratinocytes stems from a decreased ability of EZH2 to repress the *Cdkn1a* locus. Since EZH2 is also implicated in the degradation of stalled replication forks (Rondinelli et al. 2017), its functional removal in SKOs may also result in the accumulation of stalled replication forks and DNA damage, which triggers p53-dependent expression of p21 (Engeland 2022; Fischer 2017; Fischer et al. 2014). Furthermore, the loss of functional EZH2 results in decreased H3K27 trimethylation of the *Cdkn2a* locus and an increased expression of p16/Ink4a, p19/Arf and SASP factors (Ezhkova et al. 2011; Ezhkova et al. 2009; Ito et al. 2018). Another NIPP1 FHA ligand that may contribute to the observed NIPP1 SKO phenotype is KAP1 (Smith-Moore et al. 2018), which functions as a repressor of

p21 transcription (Lee et al. 2007; Okamoto et al. 2006; Santos and Gil 2014). Hence, in future studies it will be important to delineate the contribution of dysregulated NIPP1 FHA ligands, including EZH2 and KAP1, to the observed phenotype of the SKOs.

In conclusion, our previous (Verbinnen et al., 2020 a; b) and present data allow us to propose a model for the ontogenesis of the complex postnatal phenotype associated with the embryonic deletion of NIPP1. The first effect appears to be a p21-associated cell-cycle arrest of proliferating keratinocytes, which is apparent in cultured neonatal keratinocytes and in adolescent epidermis. We speculate that DNA damage, resulting from the skewed phosphoregulation of NIPP1 FHA ligands, functions as the primary trigger for the induction of p21. The reversible cell-cycle arrest of proliferating keratinocytes in adolescent SKOs accounts for the retarded onset of the 1st hair cycle and coincides with an increased expression of SASP factors. Secreted SASP factors subsequently induce or worsen senescence in neighbouring cells. SASP factors also mediate the recruitment of immune cells, leading to inflammation and hyperproliferation of remaining non-senescent proliferating keratinocytes. This accounts for the accelerated onset of the 2nd hair cycle in young adult mice. Persistent DDR signaling also results in the induction of p16/Ink4a and/or p19/Arf. A subset of senescent cells is gradually removed by recruited NK and T cells (Rao and Jackson 2016; Wang et al. 2022), resulting in the depletion of epidermal stem cells and premature aging. Senescence and, ultimately, the removal of DMBA-targeted keratinocytes, in addition to an increased expression of DNA-repair factors (Verbinnen et al., 2020a), can explain the strong resistance of adult SKOs to chemically-induced mutagenesis.

MATERIALS AND METHODS

Animals

Mice were housed in a pathogen-free animal facility under standard 12h-light/dark cycles with water and chow *ab libitum*. *Krt14-Cre* mediated CTR and SKO mice were previously described Verbinnen et al. (2020b). Both sexes were used throughout the study. Genomic DNA was extracted from ear biopsies by incubation for 1 hour at 96 °C with lysis reagent (25 mM NaOH, 0.2 mM EDTA). The extracts were neutralized with 50 mM unbuffered MOPS. Primers used for genotyping are shown in Table S3. PCR products were loaded on 2% agarose gels and images were acquired using the E-Box device (Vilber). The dorsal skin of mice was treated with 7,12-dimethylbenz[a]anthracene (DMBA) for 4 hours according to Verbinnen et al. (2020a). For the collection of tissue, animals were euthanized with an overdose of the pentobarbital Dolethal. All experimental protocols were in accordance with and approved by the Guide of Care of Experimental Animals of the KU Leuven Ethical Committee (license numbers 053/2018 and 021/2023).

Hair(-cycle) analysis

For hair-cycle analysis, the dorsal skin of mice was trimmed with an electric hair clipper (Clipper Aesculap Isis) at PD19-21 (telogen-1) (shaving experiment Figure 1b-d) or PD31 (shaving experiment Figure 1e-h) in a posterior-to-anterior direction and care was taken to avoid wounds, as described in Wang et al. (2023). For the long-term hair cycle analysis (Figure 1e-h), mice were tracked from PD 31 until PD 160, and newly grown fur was periodically shaved. The color of the shaved patch and hair regrowth was monitored and photographed daily (experiment Figure 1b-d) or every other day (experiment Figure 1e-h) until hair-coat recovery. For both hair-cycle experiments, photographs were chronologically arranged and analysed. The hair-cycle stages were determined as described by Müller-Röver et al. (2001).

Depilation experiments of dorsal skin was performed at PD21 (telogen-1). A $\pm 2\text{-cm}^2$ area of back skin was shaved and depilated using wax-strips (Veet) after anaesthesia with iso-flurane

(Iso-Vet). Mice were monitored and photographed daily for hair-coat recovery. Hair analysis (Figure 1j, k and S1g, h) is described in Supplementary Materials and Methods.

Flow cytometry

Suprabasal and basal HFSCs, BECs and PKs were analysed by flow cytometry as detailed in Supplementary Materials and Methods. Senescence-associated β -Galactosidase (SA- β -Gal) was detected in tail epidermal cells obtained from 8-week-old CTR and SKO mice by flow cytometry. Tail skin pieces were prepared and single cells obtained according to Verbinnen et al. (2020b) and stained using a CellEvent™ Senescence Green Flow Cytometry Assay Kit (Invitrogen; C10840), according to the manufacturer's instructions with some minor modifications as detailed in Supplementary Materials and Methods.

Histological, immunohistochemical and immunofluorescence analysis and biochemical procedures

Skin samples from the upper part of the back skin were taken, as recommended by Wang et al. (2023). For histological and immunohistochemical stainings on paraffin-embedded tissues, dorsal-skin sections were treated as described by Verbinnen et al. (2020b). Immunohistochemistry on frozen sections and immunofluorescence on CTR and SKO keratinocytes that were cultured for 2,4, 6 days on collagen-I/fibronectin-I coated plates were performed and analysed as described in Supplementary Materials and Methods. Total protein lysates from tail epidermis and HaCaT cells were made and subjected to Western blot analyses as described in Supplementary Materials and Methods. All antibodies are listed in Supplementary Table S4.

Isolation and growth assays of primary keratinocytes and cell culture

Keratinocytes were isolated from 1-2-day old CTR and SKO pups, seeded on collagen I/fibronectin I-coated plates, harvested and analysed as detailed in Supplementary Materials and Methods. HaCaT cells and 3T3-J2 cells were maintained and treated as described in Supplementary Materials and Methods.

RT-qPCR

Total RNA was isolated from snap-frozen mouse epidermis, FACS-purified HFSCs and BECs, PKs and HaCaT cells. cDNA synthesis and RT-qPCR were carried out as described in Supplementary Materials and Methods. All RT-qPCR primers are listed in Table S5.

Statistical analysis

All results are shown as means $\pm$ standard deviation (SD) and all data from RT-qPCR and Western Blot analysis are shown in fold change relative to one CTR value. Statistical analysis was performed using GraphPad Prism Software 8.0 (GraphPad software, Inc) or *R* version 4.2.2 (<https://www.R-project.org/>). Statistical significance was determined using an unpaired two-tailed Student's *t*-test with or without Welch's correction, or Mann-Whitney *U* test. When multiple groups or more than one variance was analysed, one-way and two-way analysis of variance ANOVA was used.

DATA AVAILABILITY STATEMENT

No large datasets were generated during the current study.

CONFLICT OF INTEREST

The authors state no conflict of interest

ACKNOWLEDGEMENTS

This work was financially supported by the Foundation Against Cancer (2020-065) and the Special Research Fund of KU Leuven (BOF C14/20/101). Annemie Hoogmartens is acknowledged for expert technical assistance. We thank Reena Chinnaraj, Vera Dermesrobian, Pradeep Kumar and Pier Andrée Pentilla of the KU Leuven Flow and Mass Cytometry Facility for their expert technical assistance.

AUTHORS CONTRIBUTIONS

Conceptualization, MJ, AVE, MB; Formal analysis, MJ, TV, IV; Funding acquisition, AVE, MB; Investigation, MJ, TV, IV, MF, KS; Methodology, MJ, TV, FW, GVdH, ZA; Project administration, MB; Supervision, AVE, MB; Revising manuscript, MJ, AVE, MB, W-H L, TV, IV, MF; Validation, MJ; Writing manuscript: MJ, AVE, MB.

REFERENCES

- Adhikari A, Davie JK. The PRC2 complex directly regulates the cell cycle and controls proliferation in skeletal muscle. *Cell Cycle*. Taylor & Francis; 2020;19(18):2373 Available from: [/pmc/articles/PMC7513841/](https://pubmed.ncbi.nlm.nih.gov/32813841/)
- Ali N, Zirak B, Rodriguez RS, Pauli ML, Truong HA, Lai K, et al. Regulatory T Cells in Skin Facilitate Epithelial Stem Cell Differentiation. *Cell*. Elsevier Inc.; 2017;169(6):1119-1129.e11 Available from: <http://dx.doi.org/10.1016/j.cell.2017.05.002>
- Alonso L, Fuchs E. The hair cycle. *J. Cell Sci*. 2006;119(3):391–3
- Beke L, Kig C, Linders JTM, Boens S, Boeckx A, Van Heerde E, et al. MELK-T1, a small-molecule inhibitor of protein kinase MELK, decreases DNA-damage tolerance in proliferating cancer cells. *Biosci. Rep. Portland Press Ltd*; 2015;35(6)
- Blank MF, Grummt I. The seven faces of SIRT7. *Transcription*. Taylor & Francis; 2017;8(2):67–74 Available from: <http://dx.doi.org/10.1080/21541264.2016.1276658>
- Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell*. 2004;118(5):635–48
- Boens S, Verbinen I, Verhulst S, Szekér K, Ferreira M, Gevaert T, et al. Brief Report: The Deletion of the Phosphatase Regulator NIPP1 Causes Progenitor Cell Expansion in the Adult Liver. *Stem Cells*. 2016;34(8):2256–62
- Van Dessel N, Boens S, Lesage B, Winkler C, Görnemann J, Van Eynde A, et al. Protein phosphatase PP1-NIPP1 activates mesenchymal genes in HeLa cells. *FEBS Lett. Federation of European Biochemical Societies*; 2015;589(12):1314–21 Available from: <http://dx.doi.org/10.1016/j.febslet.2015.04.017>
- Duverger O, Morasso MI. Epidermal patterning and induction of different hair types during mouse embryonic development. *Birth Defects Res. Part C - Embryo Today Rev*. 2009;87(3):263–72
- Engeland K. Cell cycle regulation: p53-p21-RB signaling. *Cell Death Differ*. 2022 295. Nature Publishing Group; 2022;29(5):946–60 Available from: <https://www.nature.com/articles/s41418-022-00988-z>
- Van Eynde A, Nuytten M, Dewerchin M, Keppens S, Beullens M, Carmeliet P, et al. The Nuclear Scaffold Protein NIPP1 Is Essential for Early Embryonic Development and Cell Proliferation. *Mol. Cell. Biol*. 2004;24(13):5863–74
- Ezhkova E, Lien WH, Stokes N, Pasolli HA, Silva JM, Fuchs E. EZH1 and EZH2 cogovern histone H3K27 trimethylation and are essential for hair follicle homeostasis and wound repair. *Genes Dev. Cold Spring Harbor Laboratory Press*; 2011;25(5):485 Available from: [/pmc/articles/PMC3049289/](https://pubmed.ncbi.nlm.nih.gov/213049289/)
- Ezhkova E, Pasolli HA, Parker JS, Stokes N, Su I, Hannon G, et al. Ezh2 Orchestrates Gene Expression for the Stepwise Differentiation of Tissue-Specific Stem Cells. *Cell*. Elsevier Ltd; 2009;136(6):1122–35 Available from: <http://dx.doi.org/10.1016/j.cell.2008.12.043>
- Fan T, Jiang S, Chung N, Alikhan A, Chyi-Chia CN, Lee R, et al. EZH2-dependent suppression of a cellular senescence phenotype in melanoma cells by inhibition of p21/CDKN1A expression. *Mol. Cancer Res. American Association for Cancer Research*;

- 2011;9(4):418–29 Available from: <https://aacrjournals.org/mcr/article/9/4/418/90847/EZH2-Dependent-Suppression-of-a-Cellular>
- Ferreira M, Beullens M, Bollen M, Van Eynde A. Functions and therapeutic potential of protein phosphatase 1: Insights from mouse genetics. *BBA - Mol. Cell Res.* Elsevier; 2018a;(June) Available from: <https://doi.org/10.1016/j.bbamcr.2018.07.019>
- Ferreira M, Boens S, Winkler C, Szekér K, Verbinen I, Van Eynde A, et al. The protein phosphatase 1 regulator NIPP1 is essential for mammalian spermatogenesis. *Sci. Rep.* 2017;7(1):13364 Available from: <http://www.nature.com/articles/s41598-017-13809-y>
- Ferreira M, Verbinen I, Fardilha M, Van Eynde A, Bollen M. The deletion of the protein phosphatase 1 regulator NIPP1 in testis causes hyperphosphorylation and degradation of the histone methyltransferase EZH2. *J. Biol. Chem.* 2018b;293(47):18031–9
- Fischer M. Census and evaluation of p53 target genes. *Oncogene.* Nature Publishing Group; 2017;36(28):3943–56
- Fischer M, Steiner L, Engeland K. The transcription factor p53: Not a repressor, solely an activator. *Cell Cycle.* 2014;13(19):3037–58
- Freund A, Laberge RM, Demaria M, Campisi J. Lamin B1 loss is a senescence-associated biomarker. *Mol. Biol. Cell.* 2012;23(11):2066–75
- Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of Cellular Senescence. *Trends Cell Biol.* Elsevier Ltd; 2018;28(6):436–53 Available from: <http://dx.doi.org/10.1016/j.tcb.2018.02.001>
- Heroes E, Lesage B, Görnemann J, Beullens M, Van Meervelt L, Bollen M. The PP1 binding code: a molecular-lego strategy that governs specificity. *FEBS J.* John Wiley & Sons, Ltd; 2013;280(2):584–95 Available from: <https://onlinelibrary.wiley.com/doi/full/10.1111/j.1742-4658.2012.08547.x>
- Herranz N, Gil J. Mechanisms and functions of cellular senescence. *J. Clin. Invest.* American Society for Clinical Investigation; 2018. p. 1238–46
- Ito T, Teo YV, Evans SA, Neretti N, Sedivy Correspondence JM. Regulation of Cellular Senescence by Polycomb Chromatin Modifiers through Distinct DNA Damage- and Histone Methylation-Dependent Pathways. *Cell Rep.* 2018;22:3480–92 Available from: <https://doi.org/10.1016/j.celrep.2018.03.002>
- Kimura-Ueki M, Oda Y, Oki J, Komi-Kuramochi A, Honda E, Asada M, et al. Hair cycle resting phase is regulated by cyclic epithelial FGF18 signaling. *J. Invest. Dermatol.* Elsevier Masson SAS; 2012;132(5):1338–45 Available from: <http://dx.doi.org/10.1038/jid.2011.490>
- Kiran S, Oddi V, Ramakrishna G. Sirtuin 7 promotes cellular survival following genomic stress by attenuation of DNA damage, SAPK activation and p53 response. *Exp. Cell Res.* Elsevier; 2015;331(1):123–41
- Kulaberoglu Y, Gundogdu R, Hergovich A. The Role of p53/p21/p16 in DNA-Damage Signaling and DNA Repair. *Genome Stab. From Virus to Hum. Appl.* Elsevier Inc.; 2016;243–56
- Lee J, Hoi CSL, Lilja KC, White BS, Lee SE, Shalloway D, et al. Runx1 and p21 synergistically limit the extent of hair follicle stem cell quiescence in vivo. *Proc. Natl. Acad. Sci. U. S. A.* National Academy of Sciences; 2013;110(12):4634–9 Available from:

<https://www.pnas.org/doi/abs/10.1073/pnas.1213015110>

Lee SA, Li KN, Tumber T. Stem cell-intrinsic mechanisms regulating adult hair follicle homeostasis. *Exp. Dermatol.* 2021;30(4):430–47

Lee YK, Thomas SN, Yang AJ, Ann DK. Doxorubicin Down-regulates Krüppel-associated Box Domain-associated Protein 1 Sumoylation That Relieves Its Transcription Repression on p21WAF1/CIP1 in Breast Cancer MCF-7 Cells. *J. Biol. Chem. Elsevier*; 2007;282(3):1595–606

Li G, Tang X, Zhang S, Jin M, Wang M, Deng Z, et al. SIRT 7 activates quiescent hair follicle stem cells to ensure hair growth in mice . *EMBO J.* 2020;39(18):1–17

Minnebo N, Görnemann J, O’Connell N, Van Dessel N, Derua R, Vermunt MW, et al. NIPP1 maintains EZH2 phosphorylation and promoter occupancy at proliferation-related target genes. *Nucleic Acids Res.* 2013;

Müller-Röver S, Handjiski B, Van Der Veen C, Eichmüller S, Foitzik K, McKay IA, et al. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *J. Invest. Dermatol. Blackwell Publishing Inc.*; 2001. p. 3–15

O’Connell N, Nichols SR, Heroes E, Beullens M, Bollen M, Peti W, et al. The molecular basis for substrate specificity of the nuclear NIPP1:PP1 holoenzyme. *Structure. Elsevier Ltd*; 2012;20(10):1746–56 Available from: <http://dx.doi.org/10.1016/j.str.2012.08.003>

Okamoto K, Kitabayashi I, Taya Y. KAP1 dictates p53 response induced by chemotherapeutic agents via Mdm2 interaction. *Biochem. Biophys. Res. Commun. Biochem Biophys Res Commun*; 2006;351(1):216–22 Available from: <https://pubmed.ncbi.nlm.nih.gov/17056014/>

Plikus M V. New activators and inhibitors in the hair cycle clock: Targeting stem cells state of competence. *J. Invest. Dermatol. Elsevier Masson SAS*; 2012;132(5):1321–4 Available from: <http://dx.doi.org/10.1038/jid.2012.38>

Quigley DA, Kandyba E, Huang P, Halliwill KD, Sjölund J, Pelorosso F, et al. Gene Expression Architecture of Mouse Dorsal and Tail Skin Reveals Functional Differences in Inflammation and Cancer. *Cell Rep. Elsevier B.V.*; 2016;16(4):1153–65

Rao SG, Jackson JG. SASP: Tumor Suppressor or Promoter? Yes! *Trends in Cancer. Elsevier Inc.*; 2016;2(11):676–87

Raza U, Tang X, Liu Z, Liu B. SIRT7: the seventh key to unlocking the mystery of aging. *Physiol. Rev.* 2024;104(1):253–80

Rondinelli B, Gogola E, Yücel H, Duarte AA, Van De Ven M, Van Der Sluijs R, et al. EZH2 promotes degradation of stalled replication forks by recruiting MUS81 through histone H3 trimethylation. *Nat. Cell Biol.* 2017;19(11):1371–8

Santos J, Gil J. TRIM28/KAP1 regulates senescence. *Immunol. Lett. Elsevier B.V.*; 2014;162(1):281–9 Available from: <http://dx.doi.org/10.1016/j.imlet.2014.08.011>

Savage KI, Gorski JJ, Barros EM, Irwin GW, Manti L, Powell AJ, et al. Identification of a BRCA1-mRNA Splicing Complex Required for Efficient DNA Repair and Maintenance of Genomic Stability. *Mol. Cell. Cell Press*; 2014;54(3):445–59

Schneider MR, Schmidt-Ullrich R, Paus R. The Hair Follicle as a Dynamic Miniorgan. *Curr. Biol. Elsevier Ltd*; 2009;19(3):R132–42 Available from:

<http://dx.doi.org/10.1016/j.cub.2008.12.005>

Smith-Moore S, Neil SJD, Fraefel C, Michael Linden R, Bollen M, Rowe HM, et al. Adeno-associated virus Rep proteins antagonize phosphatase PP1 to counteract KAP1 repression of the latent viral genome. *Proc. Natl. Acad. Sci. U. S. A. National Academy of Sciences*; 2018;115(15):E3529–38 Available from: <https://www.pnas.org/doi/abs/10.1073/pnas.1721883115>

Stenn KS, Paus R. Controls of hair follicle cycling. *Physiol. Rev.* 2001;81(1):449–94

Verbinnen I, Boens S, Ferreira M, Szekér K, Van Wijk L, Van Eynde A, et al. Enhanced DNA-repair capacity and resistance to chemically induced carcinogenesis upon deletion of the phosphatase regulator NIPP1. *Oncogenesis*. Springer US; 2020a; Available from: <http://dx.doi.org/10.1038/s41389-020-0214-3>

Verbinnen I, Jonkhout M, Liakath-Ali K, Szekér K, Ferreira M, Boens S, et al. Phosphatase Regulator NIPP1 Restrains Chemokine-Driven Skin Inflammation. *J. Invest. Dermatol.* 2020b;140(8):1576–88

Wang L, Lankhorst L, Bernards R. Exploiting senescence for the treatment of cancer. *Nat. Rev. Cancer* 2022 226. Nature Publishing Group; 2022;22(6):340–55 Available from: <https://www.nature.com/articles/s41568-022-00450-9>

Wang WH, Ramos R, Tai KY, Wu YS, Chang TY, Yan JY, et al. Studying Hair Growth Cycle and its Effects on Mouse Skin. *J Invest Dermatol.* 2023 Sep 1;143(9):1638–45.

Winkler C, Wu D, Rouget R, Beullens M, Van Eynde A, Bollen M. Overexpression of PP1-NIPP1 limits the repair capacity of DNA double-strand breaks. *J. Cell Sci.* 2018;(June)

Wu D, Van der Hoeven G, Claes Z, Van Eynde A, Bollen M. DNA-damage induced allosteric activation of protein phosphatase PP1:NIPP1 through Src kinase-induced circularisation of NIPP1. *FEBS J.* 2023;in press

Yu M, Mazor T, Huang H, Huang HT, Kathrein KL, Woo AJ, et al. Direct Recruitment of Polycomb Repressive Complex 1 to Chromatin by Core Binding Transcription Factors. *Mol. Cell.* Elsevier Inc.; 2012;45(3):330–43 Available from: <http://dx.doi.org/10.1016/j.molcel.2011.11.032>

Zhang N, Kaur R, Akhter S, Legerski RJ. Cdc5L interacts with ATR and is required for the S-phase cell-cycle checkpoint. *EMBO Rep.* 2009;10(9):1029–35 Available from: <http://dx.doi.org/10.1038/embor.2009.122>

Zhang X, Yu Q, Wu Y, Zhang Y, He Y, Wang R, et al. Glc7/PP1 dephosphorylates histone H3T11 to regulate autophagy and telomere silencing in response to nutrient availability. *Cell Discov.* 2023;9(1):1–25

FIGURE LEGENDS**Figure 1. Skewed hair-cycle progression in NIPP1-depleted skin.**

- a. Scheme of the synchronized first hair cycles in mice. PD20, start of hair-shaving experiment as detailed in (b); PD31, start of hair-shaving experiment as detailed in (e).
- b. Schematic representation of hair-shaving experiments of adolescent mice. The dorsal skin of CTR and SKO mice were shaved once at either PD19-21 (telogen-1, denoted as PD20) and monitored daily for hair formation until PD41. The timing of hair growth in CTRs and SKOs is indicated.
- c. Representative pictures of CTR/SKO mice from the hair-shaving experiment detailed in panel (b).
- d. Quantification of the data in panel (c). The age of hair regrowth (days) was quantified. CTR, n=6; SKO, n=5.
- e. Schematic representation of hair-shaving experiments of adult mice. Mice were shaved daily from PD31 (anagen-1) onwards and monitored until PD160. The timepoints for (dis)coloration (upper part) and hair coat formation (lower part) in CTRs and SKOs are indicated.
- f. Representative pictures of CTR/SKO mice from the hair-shaving experiment described in panel (e).
- g. Quantification of the data in panel (f). The period between the 2nd telogen (black-to-pink discoloration) and mid-2nd anagen (returning to black) was quantified in days. Quantification of the data per gender is described in Figure S1c. CTR, n=6 (4 females and 2 males); SKO, n=5 (2 females and 3 males).
- h. Quantification of the data in panel (f). The age of mice at the time of hair coat formation was quantified. Quantification of the data per gender is described in Figure S1c. CTR, n=6 (4 females and 2 males); SKO, n=5 (2 females and 3 males).

- i. CTRs and SKOs were depilated at DP21 and followed daily for hair regrowth. The bars show the days post depilation needed for initiation of hair regrowth. CTR, n=6; SKO, n=5.
- j. Quantification of the three mean hair types (Guard, Awl and Zigzag) of CTRs and SKOs at PD56 (n=3, >100 hairs per mouse).
- k. Violin plots of the length of the indicated hair types in panel (j). Long dotted line, median; small dotted line, 25 and 75 percentiles.
- l. Schematic representation of the first two hair cycles in CTR and SKO mice. PD43, start of telogen-2 in both CTR and SKO; PD80, mid-anagen-2 in SKO; PD115, mid-anagen-2 in CTR.

Results are shown as means $\pm$ SD. ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Unpaired student's T-test (d, g-j), Mann-Whitney U test (k). Ana, anagen; Cat, catagen; CTR, *Tg(Krt14-Cre/Ppp1r8^{fl/+})*; DPD, days post depilation; E, embryonic; PD, postnatal day; SKO, *Tg(Krt14-Cre/Ppp1r8^{fl/-})*; Tel, telogen.

Figure 2. SKO HFSCs show primarily delayed activation.

- a. H&E staining of dorsal skin sections at the indicated ages. Scale bar = 100 μ m.
- b. Quantification of distinct hair-cycle phases at the indicated ages. PD21, PD28 and PD77, n =4; PD56, n=6; 10 HF/mouse were analysed.
- c. Immunostaining for CD34 (green) and Ki67 (red) of mouse dorsal skin sections at the indicated ages. The pictures show the bulge region. The old bulge (OB) and new bulge (NB) are marked. DAPI (blue) was used as a nuclear counterstain. Scale bar = 100 μ m.
- d. Quantification of the data in panel (c). Number of CD34⁺/Ki67⁺ cells at the indicated ages, expressed as a percentage of all CD34⁺ bulge cells. PD21 and PD28, n =4; PD56, n=6; PD77, n = 4; 10 HF/mouse were analysed.

- e. Immunostaining for CD34 (green) and Ki67 (red) of mouse dorsal skin sections at the indicated ages. The pictures show the HG region. DAPI (blue) was used as a nuclear counterstain. Scale bar = 100 μm .
- f. Quantification of the data in panel (e). Total number of Ki67⁺ cells per HG at the indicated ages. PD21 and PD28, n =4; PD56, n=6; 3-10 HGs per mouse were analysed.
- g. Quantification of immunostainings (Figure S2g) for the pan-immune-cell marker CD45⁺ cells of mouse dorsal skin sections at the indicated ages (n=4, ≥ 10 area's per mouse). Area = 100,000 μm^2 . For each mouse, ≥ 10 pictures were analysed and the area was extrapolated to 100,000 μm^2 .

Data are represented as means $\pm$ SD (b, g) or as violin plots (d, f), which show the median and quartiles. ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (unpaired Student's T-test (b, g), Mann-Whitney U-test (d, f)). C, catagen; CTR, Tg(*Krt14-Cre/Ppp1r8^{fl/+}*); EA, early anagen; H&E, Hematoxylin and Eosin; HF, hair follicle; HG, hair germ; LA, late anagen; MA, mid anagen; NB, new bulge; OB, old bulge; PD, postnatal day; SKO, Tg(*Krt14-Cre/Ppp1r8^{fl/-}*); T, telogen.

Figure 3. Proliferation defect of primary keratinocytes from SKOs.

- a. CTR and SKO PKs were grown on collagen-I/fibronectin-I coated plates for 5 days and proliferation curves were determined by confluency measurements using IncuCyte. CTR, n=3; SKO, n=2.
- b. PKs were seeded on inactivated 3T3-J2 feeder cells and grown for 10 days to form colonies. The colonies were visualized with crystal violet. A representative picture of a colony assay is shown. CTR, n=3; SKO, n=4.
- c. Quantification of the data in panel (b). The total area covered by the colonies was quantified, as described in Materials and Methods. CTR, n=3; SKO, n=4.

- d. – e. RT-qPCR analysis of *Itga6* (d) and *Krt10* (e) transcripts in freshly isolated PKs (0 days) and PKs harvested 2 days after plating (2 days). n=6.
 - f. Flow-cytometric cell-cycle analysis with propidium iodide of PKs harvested 2 days after seeding. An overlay of a flow-cytometric plot of CTR and SKO PKs is shown, which is representative for 4 independent experiments.
 - g. Quantification of the cell-cycle phases based on flow-cytometry data illustrated in panel (f). n=4.
 - h. Immunostaining for p21 (red) in CTR and SKO PKs at 2, 4 and 6 days after plating on collagen-I/fibronectin-I coated coverslips. DAPI (blue) was used as a nuclear counterstain. Scale bar = 50 μ m.
 - i. Quantification of the data of panel (h). The number of p21-positive cells are expressed as a percentage of the total number of cells 2-6 days post seeding. n=6-8 biological replicates, with $n \geq 50$ cells per replicate.
 - j. RT-qPCR analysis of *p19/Arf* transcript levels in PKs cultured for 2-6 days on collagen-I/fibronectin-I coated plates.
- Hprt* was used as a housekeeping gene for normalization in all RT-qPCR analysis. Results are shown as means $\pm$ SD; ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, **** = $p \leq 0.0001$ (unpaired Student's T-test (c, g, j-l); 2-way ANOVA (d, e, i). CTR, Tg(*Krt14-Cre/Ppp1r8^{fl/+}*); PI, propidium iodide; SKO, Tg(*Krt14-Cre/Ppp1r8^{fl/-}*).

Figure 4. Primary keratinocytes express senescent hallmarks after NIPP1 depletion.

- a. Immunostaining for LaminB1 (green) in CTR and SKO PKs at 6 days after plating on collagen-I/fibronectin-I coated coverslips. DAPI (blue) was used as a nuclear counterstain. Scale bar = 20 μ m.

- b. Quantification of the data of panel (a). Scatter plots show the mean fluorescent intensity of LaminB1 at the nuclear envelope normalized to CTR. CTR, n=5; SKO, n=5; with $n \geq 25$ nuclei per replicate.
- c. Immunostaining for HMGB1 (green) in CTR and SKO PKs at 6 days after plating on collagen-I/fibronectin-I coated coverslips. DAPI (blue) was used as a nuclear counterstain. Scale bar = 20 μm .
- d. Quantification of the data of panel (c). Scatter plots show the nuclear mean fluorescent intensity of HMGB1 normalized to CTR. CTR, n=5; SKO, n=6; with $n \geq 27$ nuclei per replicate.
- e. Immunostaining for 53BP1 (green) in CTR and SKO PKs at 6 days after plating on collagen-I/fibronectin-I coated coverslips. DAPI (blue) was used as a nuclear counterstain. Scale bar = 20 μm .
- f. Quantification of the data of panel (e). Scatter plots show the nuclear mean fluorescent intensity of 53BP1 normalized to CTR. CTR, n=5; SKO, n=5; with $n \geq 39$ nuclei per replicate.
- g. Immunostaining for γH2AX (green) in CTR and SKO PKs at 2, 4 and 6 days after plating on collagen-I/fibronectin-I coated coverslips. DAPI (blue) was used as a nuclear counterstain. Scale bar = 20 μm .
- h. Quantification of the data of panel (g.). Scatter plots show the nuclear mean fluorescent intensity of γH2AX at the corresponding timepoints normalized to each CTR. CTR, n=5; SKO, n=6; with $n \geq 22$ nuclei per replicate.
- i. – k. RT-qPCR analysis of *Cxcl1* (i), *Il1a* (j) and *Mmp3* (k) transcript levels in PKs cultured for 2-6 days on collagen-I/fibronectin-I coated plates. n=6 (i, k); n=5 (j).

Hprt was used as a housekeeping gene for normalization in all RT-qPCR analysis. Data are represented as means $\pm$ SD (i – k) or as violin plots (b, d, f, h), which show the median and

quartiles; ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** = $p \leq 0.0001$ (Mann Whitney U-test (b, d, f, h); unpaired Student's T-test (i – k)). CTR, Tg(*Krt14-Cre/Ppp1r8^{fl/+}*); SKO, Tg(*Krt14-Cre/Ppp1r8^{fl/-}*).

Figure 5. Loss of NIPP1 in epidermal cells results in premature senescence.

- a. The expression of various cell-cycle inhibitors and SASP-factors was measured at the indicated various ages in tail epidermis by RT-qPCR. Data are represented as heatmaps relative to 3-week old CTR mice in log2 fold change (FC). n=4, see Table S1 for average $\pm$ SD and statistical test results.
- b. The protein levels of p16 and p19 in extracts of tail epidermis from 8-week old CTRs and SKOs, as visualized by immunoblotting. γ -Tubulin was used as a loading control. n=4.
- c. RT-qPCR analysis of *Cdkn2a* locus transcript levels in isolated HFSCs and BECs from tail skin of 8-week-old CTRs and SKOs, as compared to their total epidermal expression levels. n=4.
- d. Immunostaining of γ H2AX (green) in tail epidermis of 8-week-old CTR and SKO mice. DAPI (blue) was used as a counterstain. Scale bar = 25 μ m. The right panel shows the quantification of the number of γ H2AX-positive cells per mm epidermis from 10 subsequent images per animal (n=4).
- e. Flow cytometric quantification of the percentage of senescence-associated β -Galactosidase (SA- β -Gal) positive cells in epidermal tissue from tail skin of 8-week-old CTR and SKO mice (n=6). The percentage of the SA- β -Gal+ subpopulation was normalized for each experiment to the CTR group.
- f. Immunostaining for LaminB1 (green) of mouse tail skin sections at 9 months of age. DAPI (blue) was used as a nuclear counterstain. Scale bar = 50 μ m. The right panel shows the

- quantification of the mean fluorescent intensity of LaminB1 in epidermal nuclei relative to CTR. n = 4 mice for each genotype with 9-13 pictures analysed per mouse.
- g. RT-qPCR analysis for the expression of cell-cycle inhibitors and SASP-factors in back epidermis. Data are represented as heatmaps relative to 3-week-old CTR mice in log2 fold change (FC). n=4, see Table S2 for average $\pm$ SD and statistical test results.
 - h. Immunostaining of mouse dorsal skin sections at PD56 for LaminB1 (green). DAPI (blue) was used as a nuclear counterstain. Scale bar = 50 μ m. The right panel shows the quantification of the mean fluorescent intensity of LaminB1 in epidermal nuclei relative to the CTR. n = 4 mice for each genotype with 10-16 subsequent pictures analysed per mouse.
 - i. Immunostaining for HMGB1 (green) of mouse dorsal skin sections at PD56. DAPI (blue) was used as a nuclear counterstain. Scale bar = 50 μ m. The right panel shows the quantification of the mean fluorescent intensity of HMGB1 in epidermal nuclei relative to CTR. n = 4 mice for each genotype with 7-15 subsequent pictures analysed per mouse.
 - j. RT-qPCR analysis of *Cdkn1* transcript level in isolated HFSCs and BECs from dorsal skin of 3-week-old CTRs and SKOs. n=3.
 - k. – n. RT-qPCR analysis of *p16/Ink4a* (k), *p19/Arf* (l), *Cdkn1a* (m) and *Ccl2* (n) transcript levels in back epidermis of untreated and 4h-DMBA treated mice at 7-8 weeks. n=3 – 5.
- Hprt* (a, c, g, k – n) or *Actin* (j) were used as a normalization control for all RT-qPCR analysis. Results are shown as means $\pm$ SD (c, d, e, j – n) or as violin plots (f, h, i), which show the median and quartiles; # = n.d., not-detected; ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, **** = $p \leq 0.0001$ (2-way ANOVA (k – n), unpaired student's T-test (a, d, g, j), unpaired T-test with Welch's correction (e, f, h, i). BECs, basal epidermal cells; CTR, Tg(*Krt14-Cre/Ppp1r8^{fl/+}*); DMBA, dimethylbenz[a]-anthracene; HFSCs, hair follicle stem cells; SKO, Tg(*Krt14-Cre/Ppp1r8^{fl/-}*).

Figure 6. NIPP1 knockdown induces senescence in human keratinocytes.

- a. – e. RT-qPCR analysis of *IL1A* (a), *IL8* (b), *CDKN1A* (c), *INK4A* (d) and *ARF* (e) transcripts in human keratinocyte cell line HaCaT after treatment with nontargeted siRNA (CTR siRNA) or NIPP1-specific siRNA (NIPP1 siRNA) for 24h, 48h and 72h (n=3).
- f. The level of NIPP1 and p21 protein in human HaCaT cells after treatment with nontargeted siRNA (CTR siRNA) or NIPP1-specific siRNA (NIPP1 siRNA) for 24h, 48h and 72h was determined by immunoblotting. Tata-binding protein (TBP) was used as a loading control. Representative blots for 3 independent experiments are shown.
- g. Quantification of the p21 proteins levels, as shown in panel (d), and normalised to TBP levels. n=3.
- h. The level of p53, NIPP1 and p21 protein in human HaCat cells after treatment with non-targeted siRNA (CTR siRNA), NIPP1-specific siRNA (NIPP1 siRNA) and/or p53-specific siRNA (p53 siRNA) for 48h was determined by immunoblotting. β -actin was used as a loading control. Representative blots for 3 independent experiments are shown.
- i. The level of NIPP1 and LaminB1 protein in human HaCaT cells after treatment with non-targeted siRNA (CTR siRNA) or NIPP1-specific siRNA (NIPP1 siRNA) for 48h, 72h and 96h was determined by immunoblotting. β -actin was used as a loading control. Representative blots for 2 (48h) and 3 (72h, 96h) independent experiments are shown.
- j. Immunostaining for γ H2AX (red) and NIPP1 (green) in human HaCaT cells after treatment with nontargeted siRNA (CTR siRNA) or NIPP1-specific siRNA (NIPP1 siRNA) for 72 and 96 hours. DAPI (blue) was used as a nuclear counterstain. Scale bar = 20 μ m.
- k. Quantification of nuclear γ H2AX intensity shown in panel (j) relative to each CTR. $n \geq 97$ nuclei per independent experiment (n=3).

HPRT was used as a normalization control for all RT-qPCR analysis. Data are represented as means $\pm$ SD (a – e, g) or as violin plots (k), which show the median and quartiles; ns = non-

significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$ (unpaired Student's T-test).

Journal Pre-proof

FIGURE 1

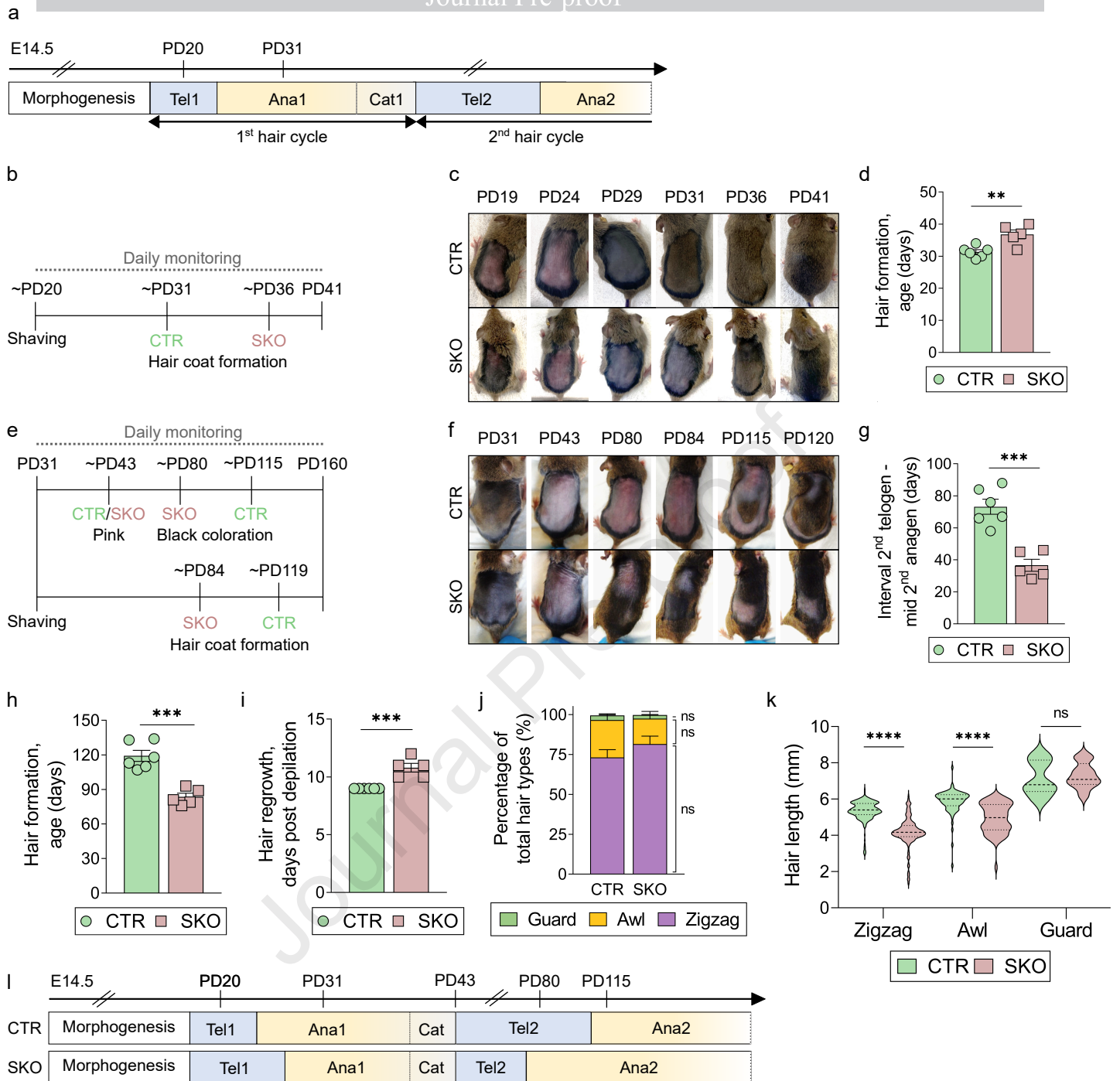


FIGURE 2

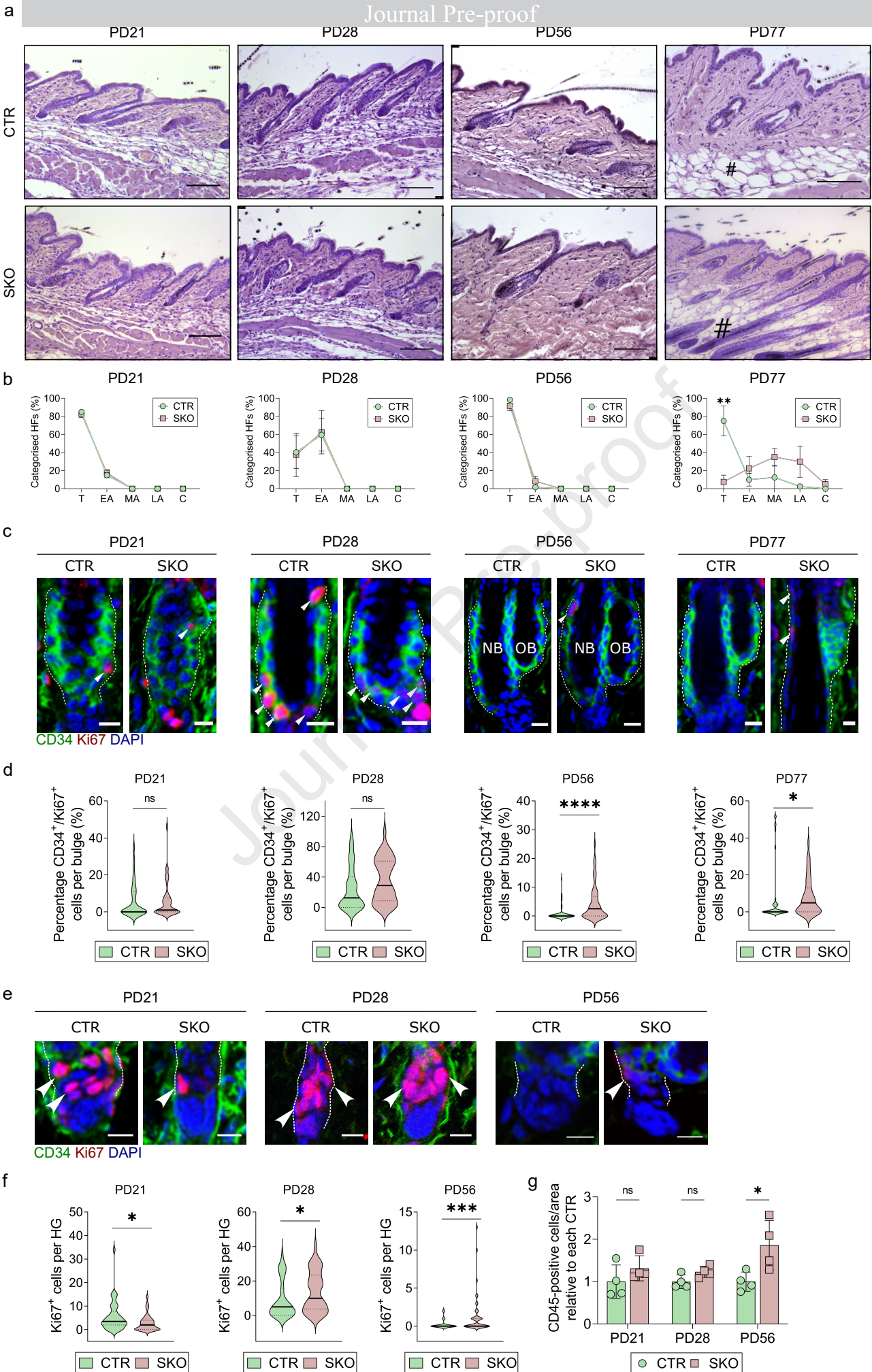


FIGURE 3

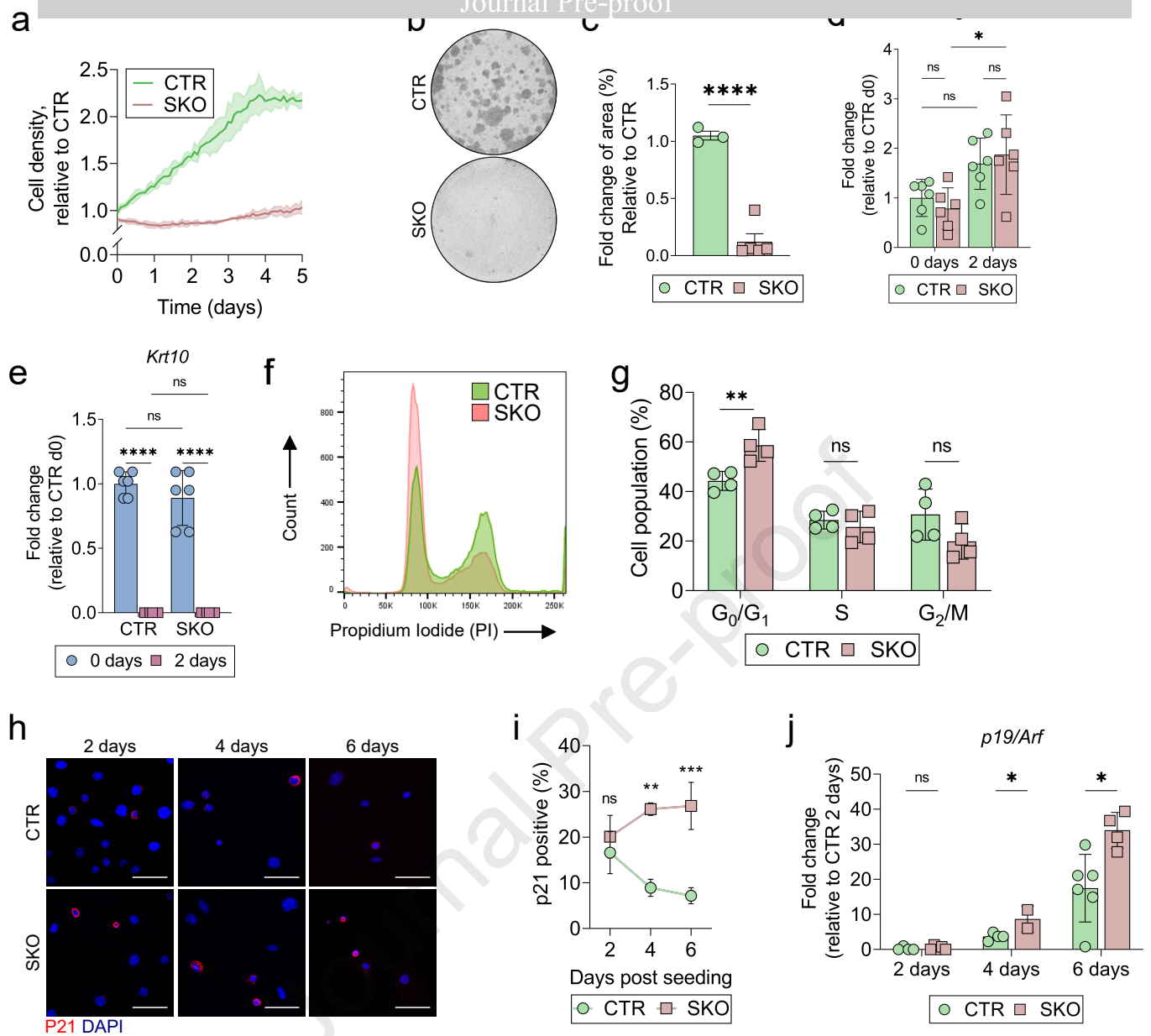


FIGURE 4

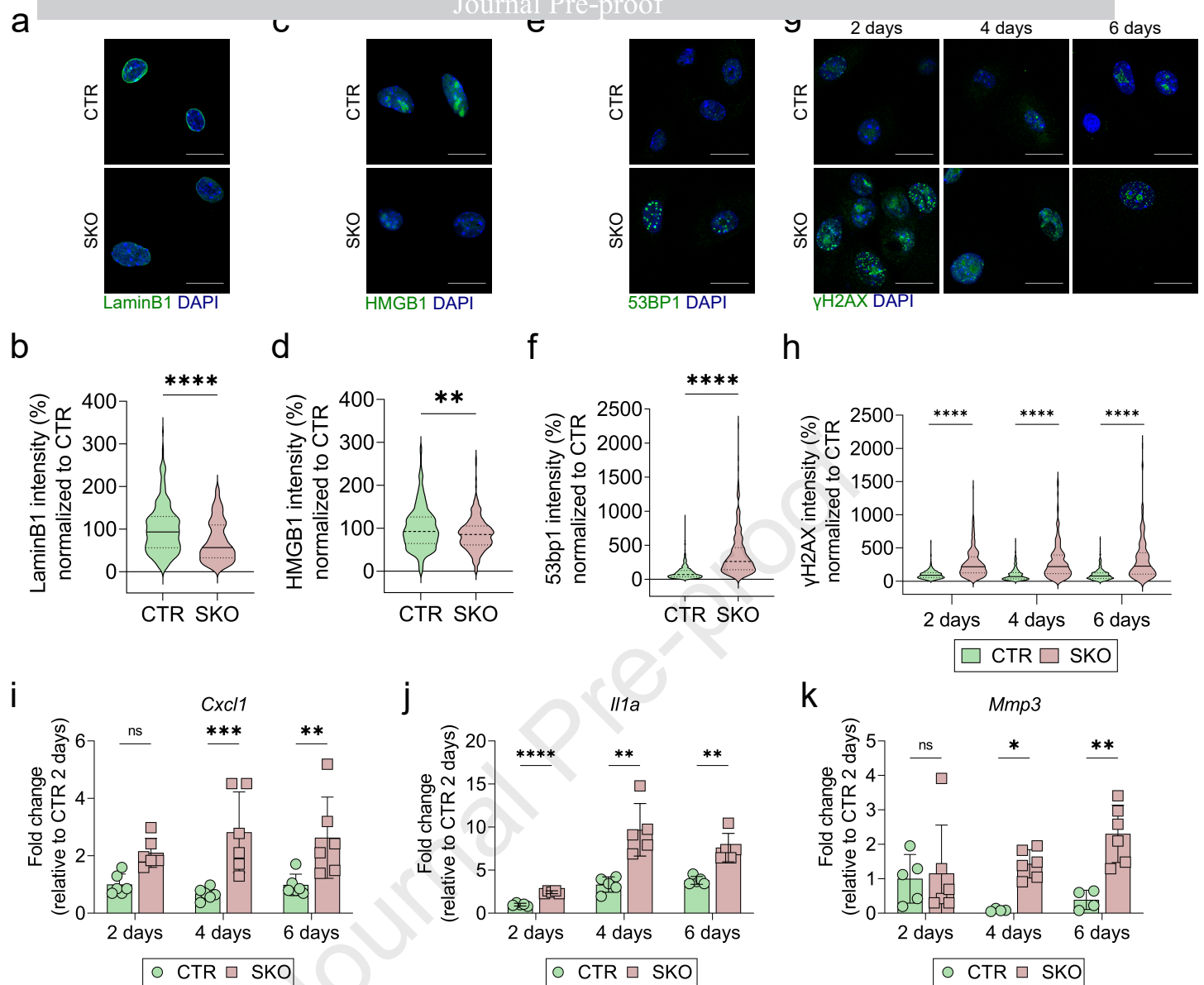


FIGURE 5

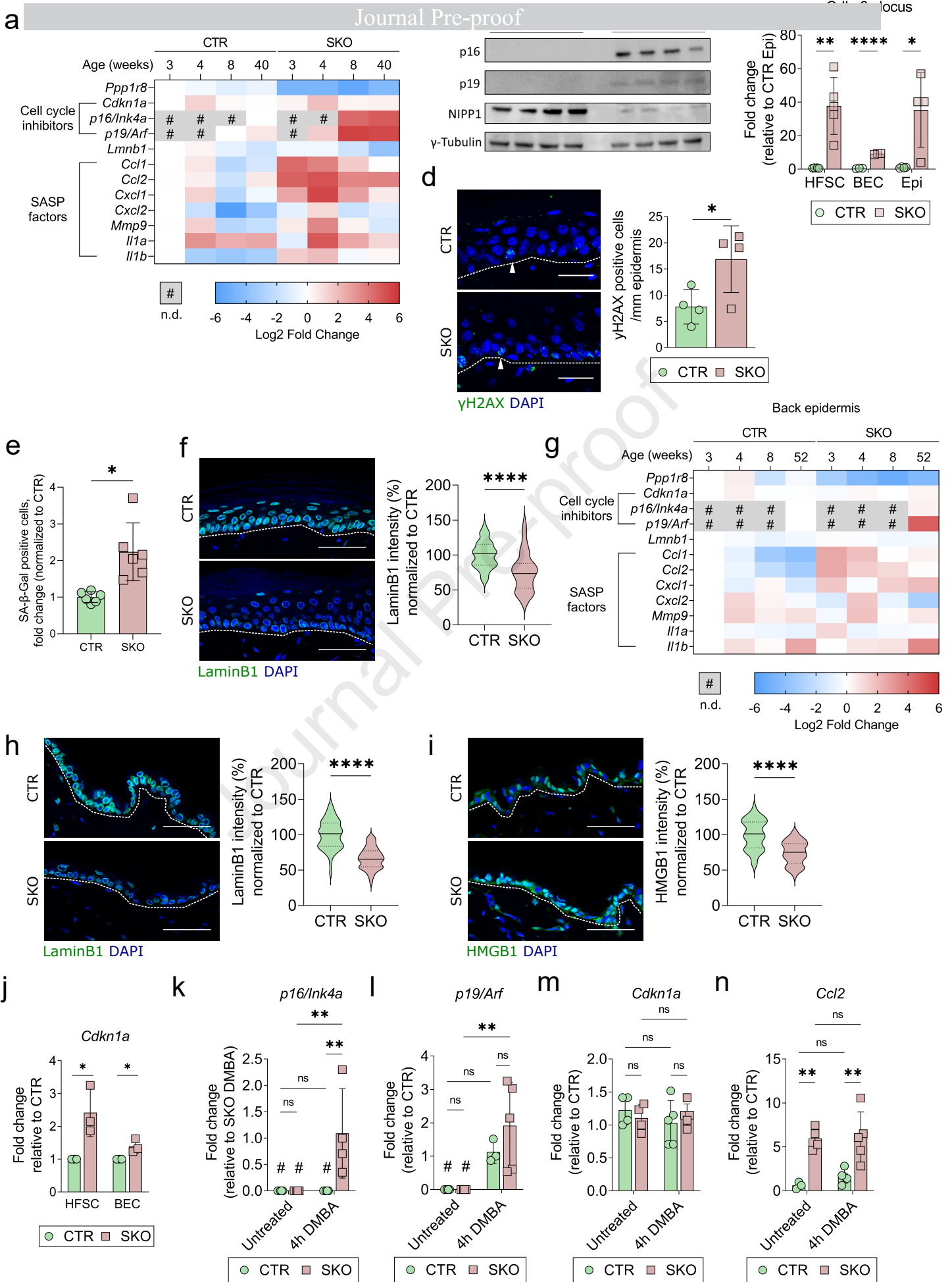
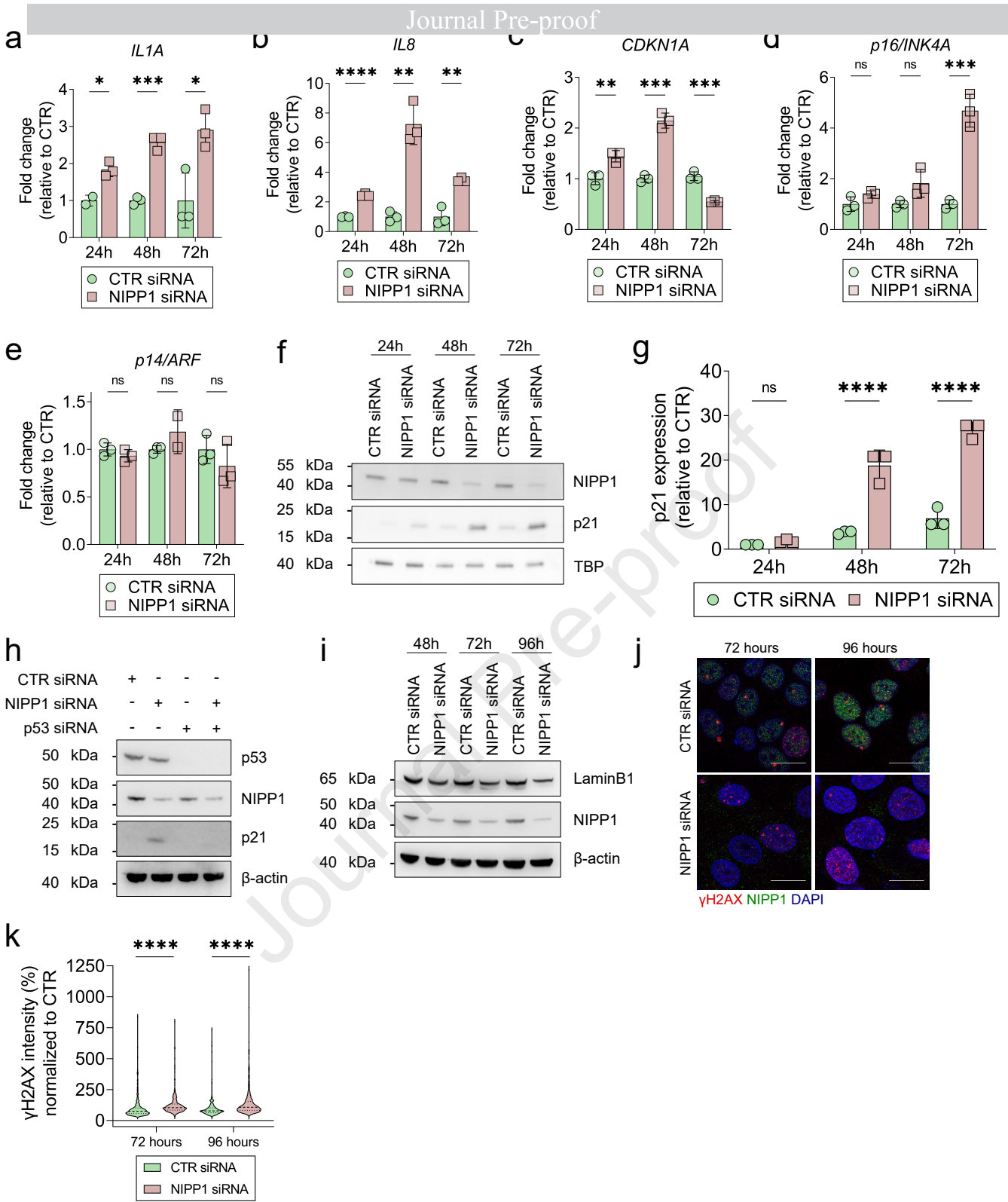


FIGURE 6



SUPPLEMENTAL FIGURES AND TABLES

Embryonic NIPP1 depletion in keratinocytes triggers a cell-cycle arrest and premature senescence in adult mice

Marloes C. M. Jonkhout¹, Tijs Vanhessche^{1*}, Mónica Ferreira^{1*}, Iris Verbinnen¹, Fabienne Withof¹, Gerd van der Hoeven¹, Kathelijne Szekér¹, Zahra Azhir¹, Wen-Hui Lien², Aleyde Van Eynde¹ and Mathieu Bollen¹

¹Laboratory of Biosignaling & Therapeutics, KU Leuven Department of Cellular and Molecular Medicine, University of Leuven, Belgium

²de Duve Institute, Faculty of Medicine, Université catholique de Louvain, Brussels, Belgium

* contributed equally

SUPPLEMENTAL INFORMATION

- Supplemental legends to the Figures S1-S6
- Supplemental Materials and Methods
- Supplemental Table S1. Mean expression levels of various transcripts in tail epidermis of CTR and SKO mice at 3, 4, 8 and 40 weeks of age.
- Supplemental Table S2. Mean expression levels of various transcripts in back epidermis of CTR and SKO mice at 3, 4, 8 and 52 weeks of age.
- Supplemental Table S3. List of primers used for genotyping.
- Supplemental Table S4. List of antibodies.
- Supplemental Table S5. List of RT-qPCR primers.
- Supplemental References

Supplemental legends to the Figures

Supplemental Figure S1. Alterations of hair-cycle progression and hair differentiation in dorsal skin of SKOs.

- a. RT-qPCR analysis of *Ppp1r8* levels in mouse skin at the indicated ages. *Hprt* was used as a housekeeping gene for normalization (n=4).
- b. NIPP1 immunostaining (green) of dorsal sections at PD21, PD28, PD56 and PD77. DAPI (blue) was used as a nuclear counterstain. Epidermis, dermis, DP and bulge are indicated in the CTR section of PD21. Scale bar = 200 μ m. e, epidermis; d, dermis; DP, dermal papilla
- c. Quantification of the data in panel (1f). The period between the 2nd telogen (black-to-pink discoloration) and mid-2nd anagen (returning to black) was quantified in days per gender. CTR, n=6 (4 females and 2 males); SKO, n=5 (2 females and 3 males).
- d. Quantification of the data in panel (1f). The age of mice at the time of hair coat formation was quantified in days per gender. CTR, n=6 (4 females and 2 males); SKO, n=5 (2 females and 3 males).
- e. Scheme of the experimental approach for depilation-induced anagen at telogen-1. Mice were depilated at PD21 and followed-up daily until DPD17. DPD, day post depilation; DPD9, the timepoint when hair regrowth was detected in CTR; DPD11, timepoint when hair regrowth was detected in SKO.
- f. Representative pictures of depilated dorsal skin of CTR/SKO of the set-up described in panel (c). CTR, n=6; SKO, n=5.
- g. Representative pictures of zigzag hairs of CTR and SKO mice at PD56. For SKOs, zigzag hairs with and without bending are shown. Scale bar = 500 μ m
- h. The proportion of (un)bended zigzag hairs on the back skin of CTRs and SKOs at PD56. Results are shown as means $\pm$ SD and are derived from 3 mice/group (76-100 zigzag hairs/mouse).

Data are represented as means $\pm$ SD; ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** = $p \leq 0.0001$ (unpaired Student's T-test (a, c, d), 2-way ANOVA (h)). CTR, Tg(*Krt14-Cre/Ppp1r8^{fl/+}*); SKO, Tg(*Krt14-Cre/Ppp1r8^{fl/-}*).

Supplemental Figure S2. Quantification of HFSCs and immune cells in CTR and SKO mice.

- a. Comparison of CD34⁺/Ki67⁺ cells at PD21 and PD28, based on quantification of data in Figure 2c, expressed as a percentage of total CD34⁺ bulge cells (n = 4; 10 HF/mouse analysed).
- b. Comparison of the total number of Ki67⁺ cells per HG at PD21 and PD28, based on quantification of data in Figure 2e, (n = 4; 10 HF/mouse analysed).
- c. Scheme of suprabasal and basal HFSCs (left), and flow-cytometric analysis of suprabasal and basal HFSCs in CTRs and SKOs at PD56 (right).
- d. Quantification of the data shown in (c). CTR, n=5; SKO, n=6.
- e. – f. Immunostaining (top) and quantification (bottom) of FoxP3 (red) of dorsal skin sections at PD56 (e) and PD77 (f). DAPI (blue) was used as a nuclear counterstain. Scale bar = 100 μ m. Quantification of FoxP3⁺ cells relative to the tissue area in the field of view and the mean intensity (a.u.) of the staining shown at PD56 (e) and PD77 (f). PD56, n=6 and PD77, n=4, ≥ 10 contiguous pictures each.
- g. Immunostaining for CD45 (green) of dorsal-skin sections at PD21, PD28 and PD56. DAPI was used as nuclear counterstain. Scale bar = 50 μ m.

Data are represented as means $\pm$ SD (d – f) or as violin plots (a, b). ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$, using unpaired Student's T-test (d – f) or Mann-Whitney U-test (a, b). BEC, basal epidermal cell; CTR, Tg(*Krt14-Cre/Ppp1r8^{fl/+}*); HG, hair germ; PD, postnatal day; SKO, Tg(*Krt14-Cre/Ppp1r8^{fl/-}*).

Supplemental Figure S3. Gene expression analysis of PKs from CTR and SKO pups.

- a. RT-qPCR analysis of *Ppp1r8* transcript levels in freshly isolated CTR and SKO keratinocytes at 0 to 6 days after seeding on collagen-I/fibronectin-I coated plates, n=6.
- b. – c. RT-qPCR analysis of *Lor* (b) and *Krt14* (c) transcript levels in freshly isolated PKs (0 days) and PKs harvested 2 days after seeding on collagen-I/fibronectin-I coated plates (2 days), n=6 (b), n ≥ 3 (c).
- d. – h. RT-qPCR analysis of *Invol* (c), *Krt1* (d), *Krt10* (e), *Krt16* (f) and *Krt17* (g) transcript levels in CTR and SKO PKs cultured for 2-, 4- or 6 days, relative to CTR 2 days. n=6.
- i. RT-qPCR analysis of *Cdkn1a* transcript in CTR and SKO PKs at 2 days after seeding on collagen-I/fibronectin-I coated plates, (n=5).
- j. RT-qPCR analysis of *p16/Ink4a* (i) transcript levels in CTR and SKO PKs cultured for 2-, 4- or 6 days on plates, relative to CTR 6 days. CTR n=6, SKO n=5, # = non-detected.

Hprt was used as a housekeeping gene for normalization. PKs were seeded on Collagen-I/fibronectin-I coated plates. Data are represented as means $\pm$ SD; ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$ (unpaired Student's T-test (a, d – i), unpaired T-test with Welch's correction (j), 2-way ANOVA (b, c)). CTR, Tg(*Krt14-Cre/Ppp1r8^{fl/+}*); PKs, primary keratinocytes; SKO, Tg(*Krt14-Cre/Ppp1r8^{fl/-}*).

Supplemental Figure S4. NIPP1-depleted primary keratinocytes express SASP markers.

- a. – d. RT-qPCR analysis of *Ccl2* (a), *Il1b* (b), *Mmp9* (c) and *Il6* (d) transcript levels in PKs cultured for 2-6 days on collagen-I/fibronectin-I coated plates (n=5-6). *Hprt* was used as a housekeeping gene for normalization in all RT-qPCR analysis. Results are shown as means $\pm$ SD; ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** = $p \leq 0.0001$ (unpaired Student's T-test). CTR, Tg(*Krt14-Cre/Ppp1r8^{fl/+}*); PKs, primary keratinocytes; SKO, Tg(*Krt14-Cre/Ppp1r8^{fl/-}*)

Supplemental Figure S5. Characterization of NIPP1 skin knockout mice and isolated epidermal cells.

- a. Pictures show CTR and SKO mice at the age of 63 weeks. n=3.
- b. RT-qPCR analysis of *Cd34* transcript levels in isolated HFSCs and BECs from tail skin of CTRs and SKOs at 8-weeks of age. *Hprt* was used as normalization control. The results are shown as means $\pm$ SD relative to CTR epidermis. N=3 (HFSCs and BECs); n=4 (epidermis)
- c. RT-qPCR analysis of *Il6* transcript levels in tail skin of CTRs and SKOs at the indicated ages (weeks). *Hprt* was used as normalization control. The results are shown as means $\pm$ SD relative to CTR of 3-week-old mice. N=4 (all, except CTR 4-weeks (n=3)).
- d. Flow cytometry plots showing the population of senescence-associated β -Galactosidase (SA- β -Gal) positive cells, gated for singlets and alive (eFluor780⁺) cells. Representative plots are shown for 3 independent experiments, with 2 mice per genotype (1 male, 1 female).
- e. RT-qPCR analysis of *Ppp1r8* transcript levels in isolated HFSCs and BECs from dorsal skin of CTRs and SKOs at PD21. *Actin* was used as normalization control. The results are shown as means $\pm$ SD relative to each CTR. N=3.
- f. – g. RT-qPCR analysis of *Cd34* (f) and *Krt14* (g) transcript levels in isolated HFSCs and BECs from dorsal skin of 3-week-old CTRs and SKOs compared to their total epidermal transcript levels. *Actin* was used as normalization control. The results are shown as means $\pm$ SD relative to CTR epidermis. N=3 (HFSCs and BECs); n=4 (epidermis).

h. RT-qPCR analysis *Ccl1* transcript levels in back epidermis of untreated and 4h-DMBA treated mice at 7-8 weeks. *Hprt* was used as normalization control and results are shown as means $\pm$ SD relative to CTR untreated. N=4.

ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$ (unpaired Student's T test (b, c, e – g), 2-way ANOVA (h)). BECs, basal epidermal cells; CTR, Tg(*Krt14-Cre/Ppp1r8^{fl/+}*); DMBA, dimethylbenz[a]-anthracene; Epi, epidermis; HFSCs, hair follicle stem cells; SKO, Tg(*Krt14-Cre/Ppp1r8^{fl/-}*).

Supplemental Figure S6. *IL6* expression in human keratinocytes is not affected by NIPP1 depletion.

RT-qPCR analysis *IL6* transcript levels in HaCat cells cultured for 24, 48 and 72 hours with scrambled siRNA (CTR siRNA) or siRNA for NIPP1 (NIPP1 siRNA). *HPRT* was used as a housekeeping gene for normalization in all RT-qPCR analysis. Results are shown as means $\pm$ SD relative to each CTR; ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** = $p \leq 0.0001$ (unpaired Student's T-test). CTR, control. n=3.

SUPPLEMENTAL MATERIALS AND METHODS

Hair analysis

For the analysis of hair type and length, hair was pulled out from the dorsal skin of euthanised 2-month-old mice. Hairs were roughly oriented and were stuck between two slides. Images were made with a Leica DMI 4000B microscope and approximately 100 hairs per animal were counted, distinguishing between zigzag, guard, awl and auchene hairs according to Botchkarev et al. (2005). Zigzag hairs were identified by the appearance of 1 medullar column.

Flow cytometry

Suprabasal and basal HFSCs of dorsal skin of 8-week old mice were analysed by flow cytometry. Basal HFSCs and BECs of dorsal skin of 3-week old mice were sorted by FACS. Dorsal skin was incubated for 30-40 minutes at 37°C in 0.25% trypsin-EDTA (Gibco). The epidermis was scraped off using a scalpel and incubated with 4% fetal bovine serum (FBS, Sigma-Aldrich) in PBS before passing through 70-µm and 40-µm cell strainers (VWR International). Next, the cells were pelleted by centrifugation (300 x *g*) for 15 minutes at 4°C, stained for 30 minutes on ice with fluorescently labelled antibodies (PE-ITGα6/CD49f, eFluor660-CD34, PE/Cy7-CD45, PE/Cy7-CD31), and washed twice with 4% FBS in PBS. To compensate for spectral overlap, UltraComp eBeads (Thermo Fisher Scientific) were single stained and recorded, and compensation was calculated. The cells were gated for single events, viability and CD45/CD31-negativity, and then analysed or sorted for ITGα6/CD34 expression. Cells with ITGα6^{high} and CD34^{high}-expression were classified as basal HFSCs, with ITGα6^{low}/CD34^{high} cells as suprabasal HFSCs, and with ITGα6^{high}/CD34^{low} cells as BECs. For quantification, cells were analysed with the BD FACSCanto II, equipped with BD FACSDiva software (version 6) (FACS core facility, KU Leuven) and further analysed with FlowJo (V10) software. For the isolation of basal HFSCs and BECs, cells were sorted in a BD FACSAriaIII, equipped with BD FACSDiva software (version 6) (FACS core facility, KU Leuven).

Basal HFSCs and BECs of tail skin of 8-week old mice were sorted by FACS according to Verbinnen et al. (2020b). Senescence-associated β-Galactosidase (SA-β-Gal) was detected in tail epidermal cells obtained from 8-week-old CTR and SKO mice by flow cytometry. Tail skin pieces were prepared and single cells obtained according to Verbinnen et al. (2020b) and stained using a CellEvent™ Senescence Green Flow Cytometry Assay Kit (Invitrogen; C10840), according to the manufacturer's instructions with some minor modifications. Cells were incubated with eBioscience Fixable Viability Dye eFluor780 (1/10.000; Invitrogen) for 30 minutes prior to fixation to stain for live and dead cells. Cells were washed in PBS containing

1% BSA and fixed in 2% PFA/PBS for 10 minutes prior to staining with 1X CellEvent™ Senescence Green Probe in the dark for 1 hour. To compensate for spectral overlap, a mix of CTR and SKO cells was single stained and recorded, and compensation was calculated. The cells were gated for single events and viability (eFluor780 negative) prior to analysis. Cells were recorded with the BD FACSCanto II using the 488nm and 633nm lasers and 530/30 and 780/60 filters, equipped with BD FACSDiva software (version 6) (FACS core facility, KU Leuven) and further analysed with FlowJo (V10) software.

For flow-cytometric analysis of primary keratinocytes (PK), CTR and SKO PKs were grown on collagen-I/fibronectin-I (C/F) coated plates for 2 days. Cells were harvested, washed with PBS, pelleted, fixed in 70% EtOH/PBS, and stored at -20°C. On the day of flow-cytometric analysis, the cells were rehydrated with PBS and incubated for 30 minutes at 37°C with PBS, supplemented with 0.1 mg/ml DNase-free RNase (Sigma) and 0.5% Triton X100. Subsequently, the cells were stained with propidium iodide (PI, Invitrogen, final concentration of 140 µg/ml) and analysed with the BD FACSCanto II, equipped with BD FACSDiva software (version 6) (FACS core facility, KU Leuven), and gated for singlets.

Histological, immunohistochemical and immunofluorescence analysis

For histological and immunohistochemical stainings on paraffin-embedded tissues, dorsal-skin sections were treated as described by Verbinnen et al. (2020b). Skin samples from the upper part of the back skin were taken, as recommended by Wang et al. (2023). Tissues were immediately fixed in 4% paraformaldehyde (PFA) in PBS, washed with PBS and maintained in 70% ethanol before dehydration in the histokinette and embedding in paraffin at the Histology & Imaging Facility (VIB Research Center for Brain & Disease Research, KU Leuven). Skin sections of 5 µm were cut using a microtome (ThermoScientific HM340E) and dehydrated through immersion in consecutively 100%, 90%, 70% and 50% ethanol. H&E stainings were carried out as described by Verbinnen et al. (2020b). For immunohistochemical staining of NIPP1, an antigen retrieval treatment was necessary and sections were treated for 7 min at 37°C with 0.25 mg/ml trypsin (Sigma-Aldrich) in 0.1% CaCl₂. Subsequently, endogenous peroxidase activity was blocked for 20 min with 0.3% H₂O₂ in MeOH. Next, the sections were permeabilised (0.5% Triton-X100 in PBS) for 30 minutes, blocked for 60 min with 10% normal goat serum (NGS, Sigma) in blocking reagent (PerkinElmer), and incubated overnight at 4°C with primary antibody. Subsequently, the sections were incubated for 60 min with goat-anti-rabbit HRP (horse radish peroxidase) (1/200) in blocking reagent and the signal was amplified with fluorescein (PerkinElmer). For γH2AX and CD45 stainings, an antigen

retrieval for 20 minutes at 100°C in citrate buffer (pH 6.0, DAKO) was performed. Prior to overnight incubation with primary antibody, the sections were permeabilised (0.5% Triton-X100 in PBS) for 5 min and blocked for 60 min at room temperature in 3% bovine serum albumin (BSA)/10% FBS/0.25% fish gelatine (Sigma-Aldrich)/0.1% Tween in PBS and incubated overnight at RT with primary anti-CD45 antibody or at 4°C with primary anti- γ H2AX antibody. Sections were incubated at room temperature for 2h with secondary goat anti-rat or goat anti-rabbit Alexa Fluor (AF) 488-conjugated antibody (Thermofisher). For both the LaminB1 and HMGB1 staining, antigen retrieval was performed for 20 min at 100°C using a Tris-EDTA-based buffer at pH 9.0, purchased from Abcam (ab93684), according to the manufacturer's instructions. Subsequently, endogenous peroxidase activity was blocked for 20 min with 0.3% H₂O₂ in MeOH. Next, the sections were permeabilized (0.1% Triton-X100 in TBS) for 30 minutes, blocked for 60 min with 10% normal goat serum (NGS, Sigma) in TBS containing 0.1% Tween-20. Sections were incubated overnight at 4°C with primary anti-LAMINB1 or anti-HMGB1 antibody in TBS containing 1% BSA and 0.1% Tween-20. Sections were incubated at room temperature for 1h with goat-anti-rabbit Alexa Fluor (AF) 488-conjugated antibody (Thermofisher) containing 1% BSA and 0.1% Tween-20. For all stainings, slides were mounted with ProLong Gold DAPI-anti fade reagent (Invitrogen). The used antibodies and their dilutions are detailed in Table S4.

For immunohistochemistry on frozen sections, dorsal-skin sections were fixed for 15 min at room temperature in PBS containing 4% paraformaldehyde (PFA), washed twice with 1M ammonium chloride, and incubated overnight at 4°C in 30% sucrose. The tissues were embedded in OCT-Tissuetek (Sakura), cut at 10 μ m using a Cryostar NX70 cryostat (VIB Research Center for Brain & Disease Research, KU Leuven), and mounted on Superfrost Plus Gold slides (Thermofisher). Depending on the used antibody, sections were fixed in methanol or acetone, blocked in 1% BSA/0.1% Tween in PBS containing 10% NGS or normal donkey serum (NDS), and incubated overnight at 4°C with the desired antibody. The used antibodies and their dilutions are detailed in Table S4. Sections were incubated for 1h with goat anti-rat AF488-conjugated, goat anti-rabbit AF546-conjugated and donkey anti-rabbit AF555-conjugated antibodies. Slides were mounted with ProLong Gold DAPI-anti fade reagent (Invitrogen). Microscopy was carried out using a Leica DMI 4000B confocal microscope and a Leica DM5500 light microscope (VIB Research Center for Brain & Disease Research, KU Leuven). Images were processed with LAS AF and ImageJ software.

For immunofluorescence analysis, CTR and SKO keratinocytes were cultured for 2, 4 and 6 days on C/F-coated coverslips in 24-well plates. Keratinocytes were washed in PBS, fixed for

15 min at 37°C in 4% PFA/PBS, and permeabilized for 15 min at room temperature in PBS plus 0.1% Triton X-100. Subsequently, the cells were blocked for 60 min at room temperature in 3% BSA/PBS and incubated overnight at 4°C with a primary antibody diluted in 3% BSA/PBS. The samples were then washed 3 times in PBS and incubated for 3 hours at room temperature with secondary Alexa Fluor-conjugated antibodies in 3% BSA/PBS. Finally, the samples were washed again 3 times in PBS and mounted on Superfrost-slides (Thermofisher) with Mowiol (Sigma-Aldrich). The used antibodies and their dilutions are detailed in Table S4. Microscopy was carried out using a Leica DMI 4000B confocal microscope.

All obtained images were processed and analysed with ImageJ software (NIH, version 1.54g). For γ H2AX, 53BP1 and HMGB1 stainings in mouse tissue, primary mouse keratinocytes and human keratinocytes, the nuclear staining intensity was measured using a written macro. Nuclei were identified based on the DAPI signal, which allowed the quantification of nuclear intensity of γ H2AX, 53BP1 and HMGB1 staining. For the LaminB1 staining in primary keratinocytes and mouse tissue, the above described macro was used with some modifications. Two masks were produced from the identified nuclei, one dilated and one eroded, in which LaminB1 staining intensity was measured. The subtraction of the intensity of the eroded from the dilated area allowed the measurement of the LaminB1 intensity within the nuclear envelope and was adjusted for the measured area.

Protein extraction

Epidermal lysates were made according to Verbinnen et al. (2020b). Briefly, epidermal tissue was resuspended in modified RIPA buffer containing 50 mM Tris-HCl at pH 7.5, 0.3 M NaCl, 0.5% Triton X-100, 0.2% sodium dodecyl sulfate (SDS), 1 mM EDTA, 0.2% sodium deoxycholate, and supplemented with protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 5 μ M leupeptin) and protein phosphatase inhibitors (25 mM sodium fluoride, 1 mM orthovanadate). After a 20-min incubation on ice the epidermal tissue was homogenized for 4 minutes on ice using a Polytron Homogenizer and centrifuged (11,337 x g) for 4 minutes at 4°C. The supernatant (sup 1) was kept and the remaining pellet was resuspended for 30 min at 37°C in nuclease buffer containing 50 mM Tris-HCl at pH 7.5, 6 units/ μ l micrococcal nuclease (Thermo Scientific), 1.5 mM CaCl₂ and a cocktail of protease and protein phosphatase inhibitors as detailed above. After re-sedimentation, the supernatant (sup) 2 was pooled with sup 1. The protein concentration of the combined supernatant was determined using the PierceTM BCA Protein Assay Kit (Thermofisher).

HaCaT cells (kindly donated by prof. Garmyn, UZ Leuven) were washed twice with PBS and lysed with modified RIPA lysis buffer and supplemented with cocktail of protease and protein phosphatase inhibitors as detailed above. Lysates were kept on ice for 10 min and then sonicated for 5 min on ice using 30-sec intervals. Next, the lysates were centrifuged for 10 min at 2415 x *g* and the supernatants were used for immunoblotting. The protein concentration of the supernatants was determined using the PierceTM BCA Protein Assay Kit (ThermoFisher). Protein lysates were further processed for SDS-PAGE and immunoblotting as mentioned earlier.

Gel electrophoresis was performed using 4-12% and 10% NuPAGE Bis-Tris gradient gels (Invitrogen) and transferred on a polyvinylidene fluoride membrane (GE Healthcare). Membranes were blocked using 5% BSA in TBS-0.1%Tween20 (for phosphosite-antibodies) or 5% non-fat dry milk in TBS plus 0.1% Tween 20. Membranes were incubated overnight at 4°C with primary antibodies and for 1h at room temperature with secondary antibodies, conjugated to HRP. HRP was detected using ECL reagent (Perkin Elmer, Life Sciences) in the ImageQuant LAS4000 imaging system (GE Healthcare). The used antibodies and their dilution are detailed in Table S4.

Cell culture

HaCaT cells were maintained in high-glucose DMEM medium, supplemented with 10% FCS, 5% Penicillin (Gibco), 5% Streptomycin (Gibco) and 5% sodium pyruvate (Sigma-Aldrich). Stealth siRNA (Invitrogen) for *PPP1R8* (5'-GGAACCUCACAAGCCUCAGCAAAUU-3'), a scrambled version (5'-GGAACUCGAACCUCCACGAACAAUU-3') and/or *TP53* SMARTPool siGENOME siRNA (Dharmacon, M-003323-03) was transfected using Lipofectamine RNAiMax (Invitrogen) in Penicillin/Streptomycin-free medium. The medium was refreshed after 24h and maintained another 24 to 72 hours prior to cell analysis.

3T3-J2 fibroblasts were kindly donated by Dr. Howard Green (Harvard College). The fibroblasts were cultured at 37°C and 5% CO₂ in a cell-culture incubator in F-medium, consisting of DMEM/F12 in a 3:1 ratio, containing 10% bovine calf serum (BCS, Gibco), 1% Penicillin (Gibco), 1% Streptomycin (Gibco) and 0.03% Sodium-Bicarbonate (Gibco). Fibroblasts were inactivated by treatment for 2h with 8 µg/ml mitomycin C (Fisher Bioreagents) and used as 'feeder' cells.

Isolation and growth assays of primary keratinocytes

Keratinocytes were isolated from 1-2-day old CTR and SKO pups as described by Blanpain et al. (2004) (5) and Lichti et al. (2008) (6). Tissues for genotyping were isolated post mortem. The keratinocytes were seeded at 37°C and 5% CO₂ in E-medium, comprising Dulbecco's Modified Eagle Medium (DMEM, Gibco) and Ham's F-12 Nutrient Mix (Gibco) in a 3:1 ratio, and supplemented with 5 mM L-Glutamine (Gibco), 1% Penicillin (Gibco), 1% Streptomycin (Gibco), 0.03% Sodium-Bicarbonate (Gibco), 15% FBS (Sigma) chelated with Chelex 100 Resin (BioRad), 5 µg/ml insulin (Sigma-Aldrich), 5 µg/ml transferrin (Sigma-Aldrich), 20 pM 3,3',5-triiodo-L-thyronine (Sigma-Aldrich), 1 nM cholera toxin (Sigma-Aldrich) and 0.42 µg/ml hydrocortisone (Millipore). The keratinocytes were kept on collagen I (ChemCruz) / fibronectin I (Sigma) (C/F)-coated plates and either harvested at the indicated time points for RNA extraction or for immunofluorescence staining.

To perform colony-formation assays, approximately 1×10^4 keratinocytes were plated in triplicate on 6-well plates in E-medium with inactivated feeder cells. The medium was refreshed every 2 days. After 10 days, the colonies were fixed for 2 hours in 2.5% glutaraldehyde and 0.2% crystal violet (w/v), washed three times with distilled water, and imaged using the digitalization option on the ImageQuant LAS Image4000 imaging system (GE Healthcare). The colony area was measured using the 'ColonyArea' macro in ImageJ.

The proliferation of primary keratinocytes was measured with IncuCyte ZOOM (Sartorius). Briefly, keratinocytes were seeded in four dilutions ranging from 8.889 cells to 50.000 cells per well in triplicate on C/F coated 96-well plates. Comparable starting confluences were used for analysis. Image scheduling, collection and analysis were conducted with the IncuCyte ZOOM and IncuCyte ZOOM software (EssenBioscience). Plates were imaged every 2 hours with a 10x objective in the phase-contrast channel. Cellular confluency was measured in the phase-contrast channel using the standard Basic Analyzer processing definition of the IncuCyte Zoom software, with a segmentation adjustment of 0.8. The confluence of SKO PKs was calculated relative to the CTR values and plotted over time.

RT-qPCR

RNA isolation, cDNA synthesis and quantitative real-time-PCR (RT-qPCR) were carried out as described by Verbinnen et al. (2020b), with slight modifications. RT-qPCR reactions were performed using the Rotor-Gene Q instrument (Corbett Research) with 1.2 µl cDNA and Apex qPCR GREEN Master Mix with Low ROX (Gentaur) according to manufacturer's instructions. The measured Ct-values were analysed with the $2^{-\Delta\Delta C_t}$ method and normalized to the

housekeeping genes *Hprt*, *Actin* or *HPRT*, as indicated. All used RT-qPCR primers were manufactured at Integrated DNA Technologies and the sequences are shown in Table S5.

Journal Pre-proof

Supplemental Table S1. Mean expression levels of various transcripts in tail epidermis of CTR and SKO mice at 3, 4, 8 and 40 weeks of age. *Hprt* was used as a housekeeping gene for normalization. Data are represented as means $\pm$ SD and normalized to CTR 3 weeks (n=4); n.d., not detectable; ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$ (unpaired Student's T-test, SKO compared to CTR for each age).

Group	CTR				SKO			
Age (weeks)	3	4	8	40	3	4	8	40
	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV
<i>Ppp1r8</i>	1 $\pm$ 0,0566	0,7622 $\pm$ 0,0982	0,4592 $\pm$ 0,0391	0,5861 $\pm$ 0,0614	0,0382 $\pm$ 0,0068 ****	0,0394 $\pm$ 0,0153 *** *	0,0217 $\pm$ 0,0096 *** *	0,0471 $\pm$ 0,0372 *** *
<i>Cdkn1a</i>	1 $\pm$ 0,1287	2,4573 $\pm$ 0,6176	1,1162 $\pm$ 0,1914	1,0114 $\pm$ 0,0344	1,2357 $\pm$ 0,2028 ns	3,5950 $\pm$ 0,3642 *	1,2740 $\pm$ 0,1608 ns	1,3417 $\pm$ 0,4207 ns
<i>Ink4a</i>	n.d.	n.d.	n.d.	1,0000 $\pm$ 0,3626	n.d.	n.d.	20,3778 $\pm$ 13,7598 *	27,6089 $\pm$ 16,4180 *
<i>Arf</i>	n.d.	n.d.	1,0000 $\pm$ 0,3528	1,6171 $\pm$ 0,2884	n.d.	2,7639 $\pm$ 1,1213 **	47,2015 $\pm$ 27,9402 *	40,0252 $\pm$ 17,4550 **
<i>Lmnbl</i>	1 $\pm$ 0,2514	1,2383 $\pm$ 0,4327	1,0000 $\pm$ 0,1255	0,5615 $\pm$ 0,1181	0,6981 $\pm$ 0,1089 ns	0,6865 $\pm$ 0,1089 ns	0,5692 $\pm$ 0,1027 **	0,1391 $\pm$ 0,0837 ***
<i>Ccl1</i>	1 $\pm$ 0,3411	1,5982 $\pm$ 1,2426	0,1802 $\pm$ 0,1065	0,3435 $\pm$ 0,1367	19,8196 $\pm$ 6,0081 ***	12,6916 $\pm$ 3,7518 **	2,9260 $\pm$ 1,5927 *	1,0258 $\pm$ 0,2089 **
<i>Ccl2</i>	1 $\pm$ 0,3171	1,4058 $\pm$ 0,7954	0,2816 $\pm$ 0,2297	1,5782 $\pm$ 0,5246	23,9069 $\pm$ 12,4112 *	32,2153 $\pm$ 19,6928 *	11,6772 $\pm$ 6,6145 *	12,3835 $\pm$ 9,5018 ns
<i>Cxcl1</i>	1 $\pm$ 0,6381	2,4862 $\pm$ 0,7843	0,1779 $\pm$ 0,1179	0,6807 $\pm$ 0,5600	6,1758 $\pm$ 2,6213 **	40,9276 $\pm$ 34,0342 ns	6,7774 $\pm$ 5,6557 ns	1,5592 $\pm$ 1,0860 ns
<i>Cxcl2</i>	1 $\pm$ 1,3432	0,2566 $\pm$ 0,1779	0,0156 $\pm$ 0,0045	0,0828 $\pm$ 0,0593	0,5053 $\pm$ 0,4583 ns	2,1441 $\pm$ 2,2958 ns	0,2736 $\pm$ 0,3055 ns	0,4483 $\pm$ 0,4561 ns
<i>Mmp9</i>	1 $\pm$ 0,1804	2,6384 $\pm$ 0,7574	0,1646 $\pm$ 0,0584	0,2357 $\pm$ 0,0774	1,4960 $\pm$ 0,4943 ns	11,5304 $\pm$ 5,2322 *	1,2996 $\pm$ 0,2770 ***	0,3751 $\pm$ 0,2206 Ns
<i>Il1a</i>	1 $\pm$ 0,2424	8,7856 $\pm$ 7,3135	5,2428 $\pm$ 1,0159	6,0175 $\pm$ 1,1592	0,4691 $\pm$ 0,5644 ns	29,1680 $\pm$ 16,7309 ns	5,8639 $\pm$ 4,2168 ns	2,3273 $\pm$ 0,7626 **
<i>Il1b</i>	1 $\pm$ 0,8753	0,1211 $\pm$ 0,0167	0,0847 $\pm$ 0,0298	0,1145 $\pm$ 0,0361	2,7720 $\pm$ 1,5335 ns	3,8624 $\pm$ 2,2568 *	0,7687 $\pm$ 0,4190 *	1,1971 $\pm$ 1,4350 ns

Supplemental Table S2. Mean expression levels of various transcripts in back epidermis of CTR and SKO mice at 3, 4, 8 and 52 weeks of age. *Hprt* was used as a housekeeping gene for normalization. Data are represented as means $\pm$ SD and normalized to CTR 3 weeks (n=4); n.d., not detectable; ns = non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p \leq 0.0001$ (unpaired Student's T-test, SKO compared to CTR for each age).

Group	CTR				SKO			
Age (weeks)	3	4	8	52	3	4	8	52
	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV	Average $\pm$ STDEV
<i>Ppp1r8</i>	1 $\pm$ 0,3358	1,4380 $\pm$ 0,4829	0,2771 $\pm$ 0,0961	0,7667 $\pm$ 0,1641	0,1134 $\pm$ 0,0796 **	0,0466 $\pm$ 0,0489 **	0,0281 $\pm$ 0,0242 **	0,0474 $\pm$ 0,0159 ***
<i>Cdkn1a</i>	1 $\pm$ 0,3129	1,3483 $\pm$ 0,9019	0,9702 $\pm$ 0,3748	0,8870 $\pm$ 0,2005	0,8107 $\pm$ 0,1635 ns	1,1539 $\pm$ 0,2832 ns	0,9991 $\pm$ 0,4132 ns	1,2736 $\pm$ 0,4073 ns
<i>Ink4a</i>	n.d.	n.d.	n.d.	1,0000 $\pm$ 2,0000	n.d.	n.d.	n.d.	1,3195 $\pm$ 2,6390 ns
<i>Arf</i>	n.d.	n.d.	n.d.	1,0000 $\pm$ 2,0000	n.d.	n.d.	n.d.	25,4027 $\pm$ 22,6839 ns
<i>Lmnbl1</i>	1 $\pm$ 0,5358	0,8032 $\pm$ 0,5155	0,5812 $\pm$ 0,3432	0,7187 $\pm$ 0,0831	1,0702 $\pm$ 0,4876 ns	0,8675 $\pm$ 0,2968 ns	0,7132 $\pm$ 0,2633 ns	0,7432 $\pm$ 0,0764 ns
<i>Ccl1</i>	1 $\pm$ 0,3965	0,5359 $\pm$ 0,3746	0,1023 $\pm$ 0,0554	0,0627 $\pm$ 0,0814	5,8100 $\pm$ 2,3399 **	3,7378 $\pm$ 3,0000 ns	0,9903 $\pm$ 0,3382 **	1,3552 $\pm$ 1,0571 ns
<i>Ccl2</i>	1 $\pm$ 1,1976	0,6399 $\pm$ 0,5824	0,2253 $\pm$ 0,1442	0,1078 $\pm$ 0,0980	5,9053 $\pm$ 0,3342 ***	2,7087 $\pm$ 1,8157 ns	2,1966 $\pm$ 0,4702 ***	1,0649 $\pm$ 0,6137 *
<i>Cxcl1</i>	1 $\pm$ 0,6590	0,5244 $\pm$ 0,5031	1,1763 $\pm$ 0,6305	0,3571 $\pm$ 0,0371	3,1252 $\pm$ 1,5487 *	0,8282 $\pm$ 0,3288 ns	2,6586 $\pm$ 1,2266 ns	3,8430 $\pm$ 4,8286 ns
<i>Cxcl2</i>	1 $\pm$ 0,9612	2,3647 $\pm$ 2,7952	1,2455 $\pm$ 0,9495	0,7959 $\pm$ 0,9795	0,3978 $\pm$ 0,4395 ns	3,1302 $\pm$ 3,4430 ns	0,5804 $\pm$ 0,0624 ns	0,1234 $\pm$ 0,0353 ns
<i>Mmp9</i>	1 $\pm$ 0,5219	2,5467 $\pm$ 1,5260	1,4153 $\pm$ 0,9567	1,8597 $\pm$ 0,6513	0,7564 $\pm$ 0,2966 ns	3,5055 $\pm$ 0,9491 ns	1,0437 $\pm$ 0,3105 ns	1,6111 $\pm$ 0,4689 Ns
<i>Il1a</i>	1 $\pm$ 0,4407	0,7682 $\pm$ 0,3765	0,6710 $\pm$ 0,3471	0,5819 $\pm$ 0,4125	2,2478 $\pm$ 0,9262 ns	0,6183 $\pm$ 0,5128 ns	0,7527 $\pm$ 0,2785 ns	0,8355 $\pm$ 0,4448 ns
<i>Il1b</i>	1 $\pm$ 0,1966	1,8900 $\pm$ 0,6360	1,1197 $\pm$ 0,3075	5,9869 $\pm$ 8,0091	1,0089 $\pm$ 0,2453 ns	1,6874 $\pm$ 0,9055 ns	1,8306 $\pm$ 0,6700 ns	10,9905 $\pm$ 10,5695 ns

Supplemental Table S3. List of primers used for genotyping

PCR		Sequence (5'→ 3')
Krt14	Forward primer	CTT GCG AAC CTC ATC ACT CG
	Reverse primer	AGG GAT CTG ATC GGG AGT TG
KO	Forward primer	CCT CAG CAG ATA GCC CAC GG
	Reverse primer	CGC ATC GCC TTC TAT CGC CTT CTT GAC
Neodel	Forward primer	CCA CCC TCT CCT TTA CTT TGT CTT C
	Reverse primer	GGA GAG GAG TAA TGA GAG GAG TTG TG

Supplemental Table S4. List of antibodies.

Target(-conjugate)	Clone	Catalog number	Supplier	Dilution	Method
CD34-eFluor660	RAM34	50-0341-82	Thermo Fisher	1/100	FC
CD49f/ITGa6-PE	GoH3	12-0495-82	Thermo Fisher	1/500	FC
CD45-PE.Cy7	30-F11	103114	BioLegend	1/50	FC
CD31-PE.Cy7	390	561410	BD Pharmingen	1/50	FC
DAPI		D1306	Thermo Fisher	0.1 µg/ml	FC
eBioscience					
Fixable Viability			Invitrogen	1/10000	FC
Dye-eFluor780					
Propidium Iodide		P3566	Invitrogen	1/10	FC
γH2AX(ph-Ser139)	20E3	9718	Cell Signaling Technologies	1/50	IHC-FFPE
CD45	30-F11	553076	BD Biosciences	1/100	IHC-FFPE
HMGB1	EPR3507	ab79823	Abcam	1/300	IHC-FFPE
LaminB1	EPR22165-121	ab229025	Abcam	1/1000	IHC-FFPE
NIPP1		HPA027452	Sigma	1/100	IHC-FFPE
CD34	RAM34	14-0341-82	Thermo Fisher	1/50	IHC-Fr
FoxP3	D6O8R	12653S	Cell Signaling Technologies	1/100	IHC-Fr
Ki67	SP6	ab16667	Abcam	1/250	IHC-Fr
γH2AX(ph-Ser139)	JBW301	05-636	Millipore	1/200; 1/100	IF
53bp1		NB100-304	Novus Biologicals	1/1000	IF
HMGB1	EPR3507	ab79823	Abcam	1/250	IF
LaminB1	EPR22165-121	ab229025	Abcam	1/1000	IF
NIPP1		HPA027452	Sigma	1/100	IF
p21		orb31546	Bio-Orbyt	1/100	IF
DAPI				1/10000	IF
β-actin	AC-15	sc-69879	Santa Cruz	1/2000; 1/5000	WB
γ-Tubulin		ab11317	Abcam	1/2000	WB
Laminb1	EPR22165-121	ab229025	Abcam	1/1000	WB
NIPP1		HPA027452	Sigma	1/1000	WB
p16	EPR20418	ab2111542	Abcam	1/1000	WB
p19	5-C3-1	NB200-174	Novus Biologicals	1/1000	WB
p21	12D1	2947S	Cell Signaling Technologies	1/1000	WB
p53		9282S	Cell Signaling Technologies	1/1000	WB
TATA binding protein		ab51841	Abcam	1/5000	WB
γ-Tubulin		ab11317	Abcam	1/2000	WB

FC, flow cytometry; FFPE, Formalin-Fixed Paraffin-Embedded; Fr, frozen, cryosections; IHC, immunohistochemistry; WB, Western blot

Journal Pre-proof

Supplemental Table S5. List of RT-qPCR primers.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
<i>Actin</i>	ACCAACTGGGACGACATGGAGAAGA	TACGACCAGAGGCATACAGGGACA
<i>Ccl1</i>	CCGTGTGGATACAGGATGTT	GAAGCTCTTTCTTCAAGGTGTTT
<i>Ccl2</i>	CTGGAGCATCCACGTGTT	GCCTACTCATTGGGATCATCTT
<i>Cd34</i>	ACCAGCCATCTCAGAGACTAT	GTAGTAGGCAGTATGCCAGTTG
<i>Cdkn2a locus</i>	GCTGGGTGGTCTTTGTGTA	TTAGCTCTGCTCTTGGGATTG
<i>Cxcl1</i>	TTCACCTCAAGAACATCCAGAG	AAGCCTCGCGACCATTC
<i>Cxcl2</i>	TCAAGAACATCCAGAGCTTGAG	CTTCAGGGTCAAGGCCAACT
<i>Hprt</i>	CTGGTGAAAAGGACCTCTCG	TGAAGTACATTATAGTCAAGGGCA
<i>Hprt #2</i>	GGACTGATTATGGACAGGACTG	GCTCTTCAGTCTGATAAAATCTAC
<i>HPRT</i>	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
<i>Il1a</i>	TCAACCAAATATATATATCAGGATGTGG	CGAGTAGGCATACATGTCAAATTTTAC
<i>IL1A</i>	TGTATGTGACTGCCCAAGATGAAG	AGAGGAGGTGGTCTCACTACC
<i>Il1b</i>	TGGACCTTCCAGGATGAGGACA	GTTTCATCTCGGAGCCTGTAGTG
<i>IL1B</i>	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
<i>Il6</i>	AACAGTGTGGGAAGCAAGTCCA	ATCGAAGCTGGAAGTGCAGCAA
<i>IL6</i>	AGCCACTCACCTCTTCAGAACGAA	CAGTGCCTCTTTGCTGCTTTTACA
<i>IL8</i>	GAGAGTGATTGAGAGTGGACCAC	CACAACCCTCTGCACCCAGTTT
<i>Invol</i>	GCCTTCTCCCTCCTGTGAGT	CTTGAGAGGTCCCTGAACCA
<i>Itga6</i>	AAGGTTTGGAGCAGATTGTTT	TTTCAGGAAGTTCCTGTTTCT
<i>Lor</i>	TCACTCATCTTCCCTGGTGCTT	GTCTTTCCACAACCCACAGGA
<i>Krt1</i>	CAGCAGGTAGACACCACAAC	ACTCATTCTCTGCGTTGGTC
<i>Krt10</i>	GACAACTGACAATGCCAACG	CTTTAGGTAGGCCAGCTCTTC
<i>Krt14</i>	CGCCGCCCTGGTGTGG	ATCTGGCGGTTGGTGGAGGTCA
<i>Krt16</i>	GTGGCCTCTAACAGTGATCTAAT	TTTGGTCTCTTCTAGGCTGTTC
<i>Krt17</i>	CAGGCTGGGGTCAGCTAGT	CCAAAGTTGCCTCCATAACC
<i>Lmnbl</i>	GAGGCGGCACTAACTCTAAG	CTCATGCTTCCCTTGTCTC
<i>Mmp3</i>	CTATGCACCTGGACCAGGGA	AGAGAGATGGAAACGGGACAAG
<i>Mmp9</i>	CAGCCAACTATGACCAGGATAA	TCGCTGGTACAGGAAGAGTA
<i>p16/Ink4a</i>	AATCTCCGCGAGGAAAGC	AGAAGAGCTGCTACGTGAAC
<i>P16/INK4A</i>	CCCAACGCACCGAATAGTTA	CAGGTACCGTGCGACATC
<i>p19/Arf</i>	CTTGGTCACTGTGAGGATTCAG	CACCGTAGTTGAGCAGAAGAG
<i>P19/ARF</i>	ATGGTGCGCAGGTTCTT	CATCATGACCTGGTCTTCTAGG
<i>p21/Cdkn1a</i>	CCCTCACTCTGTGTGTCTTAAT	TTTAAGTTTGGAGACTGGGAGAG
<i>P21/CDKN1A</i>	CCTCATCCCGTGTTCTCCTTT	GTACCACCCAGCGGACAAGT
<i>Ppp1r8</i>	TACATCTGGATGTGGTGAAAGG	GGTCGATAGTGAAGTCACACAG

Supplemental References

Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell*. 2004;118(5):635–48.

Botchkarev VA, Fessing MY. Edar Signaling in the Control of Hair Follicle Development. *J Invest Dermatol Symp Proc*. 2005 Dec 1;10(3):247–51.

Lichti U, Anders J, Yuspa SH. Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice. *Nat Protoc*. 2008;3(5):799–810.

Müller-Röver S, Handjiski B, Van Der Veen C, Eichmüller S, Foitzik K, McKay IA, et al. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. Vol. 117, *Journal of Investigative Dermatology*. Blackwell Publishing Inc.; 2001. p. 3–15.

Verbinnen I, Boens S, Ferreira M, Szekér K, Van Wijk L, Van Eynde A, et al. Enhanced DNA-repair capacity and resistance to chemically induced carcinogenesis upon deletion of the phosphatase regulator NIPP1. *Oncogenesis* [Internet]. 2020a; Available from: <http://dx.doi.org/10.1038/s41389-020-0214-3>

Verbinnen I, Jonkhout M, Liakath-Ali K, Szekér K, Ferreira M, Boens S, et al. Phosphatase Regulator NIPP1 Restrains Chemokine-Driven Skin Inflammation. *J Invest Dermatol*. 2020b;140(8):1576–88.

Wang WH, Ramos R, Tai KY, Wu YS, Chang TY, Yan JY, et al. Studying Hair Growth Cycle and its Effects on Mouse Skin. *J Invest Dermatol*. 2023 Sep 1;143(9):1638–45.