Cancer Letters 442 (2019) 299–309

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

DNp73-induced degradation of tyrosinase links depigmentation with EMT-driven melanoma progression

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ARTICLE INFO

Keywords:
Tyrosinase degradation
p73
Epithelial-mesenchymal transition
Diagnostic signature
Melanoma progression

ABSTRACT

Melanoma is an aggressive cancer with poor prognosis, requiring personalized management of advanced stages and establishment of molecular markers. Melanomas derive from melanocytes, which specifically express tyrosinase, the rate-limiting enzyme of melanin-synthesis. We demonstrate that melanomas with high levels of DNp73, a cancer-specific variant of the p53 family member p73 and driver of melanoma progression show, in contrast to their less-aggressive low-DNp73 counterparts, hypopigmentation in vivo. Mechanistically, reduced melanin-synthesis is mediated by a DNp73-activated IGF1R/PI3K/AKT axis leading to tyrosinase ER-arrest and proteasomal degradation. Tyrosinase loss triggers reactivation of the EMT signaling cascade, a mesenchymal-like cell phenotype and increased invasiveness. DNp73-induced depigmentation, Slug increase and changes in cell motility are recapitulated in neural crest-derived melanophores of *Xenopus* embryos, underscoring a previously unnoticed physiological role of tyrosinase as EMT inhibitor. This data provides a mechanism of hypopigmentation accompanying cancer progression, which can be exploited in precision diagnosis of patients with melanoma-associated hypopigmentation (MAH), currently seen as a favorable prognostic factor. The DNp73/IGF1R/Slug signature in colorless lesions might aid to clinically discriminate between patients with MAH-associated metastatic disease and those, where MAH is indeed a sign of regression.

1. Introduction

Melanoma is a skin cancer type originating from melanocytes, that produce melanin-synthetic enzymes, mainly tyrosinase (TYR), as well as its downstream-acting enzymes tyrosinase-related protein 1 (TRP-1) and dopachrome tautomerase (DCT) [1], which lead to step-wise conversion of tyrosine to melanin. Tyrosinase, a type I membrane-bound melanosomal glycoprotein enzyme, catalyzes hydroxylation of L-tyrosine and oxidation of L-DOPA, the rate limiting step of the melanin synthesis cascade. Thus, it prevents long-term accumulation of the melanin precursors that act as deleterious reactive oxygen species (ROS) and uses them as substrates to produce melanin, protecting cells from UVR-induced damage [2].

Correct protein folding and post-translational processing of tyrosinase ensures enzyme stability and activity. To this end, the 60-kDa core polypeptide is transported cotranslationally to the endoplasmic reticulum (ER). There, it undergoes addition of N-linked glycans, producing a 70-kDa immature form. The N-linked glycans are trimmed by ER glucosidases I and II, and the resulting monoglucosylated oligomannosidic glycans interact with ER-resident chaperone molecules for correct folding. At this stage, the immature forms are sensitive to endoglycosidase H (Endo-H), which cleaves asparagine-linked mannose-rich oligosaccharides added in the ER, but not the complex oligosaccharides from glycoproteins that are added later in the Golgi apparatus. In Golgi, the oligomannosidic N-linked glycans are processed to complex glycans, which finally produce an 80-kDa protein, resistant to Endo-H digestion. ER-retention followed by accelerated degradation of the immature, misfolded forms has been described for albinos who bear loss-of-function TYR mutant variants [3,4]. However, ER-arrest and proteasome degradation has been also reported for wild-type tyrosinase in some melanoma cell lines [5].

Tyrosinase is specifically expressed, together with other

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https://doi.org/10.1016/j.canlet.2018.11.009
Received 15 August 2018; Received in revised form 30 October 2018; Accepted 7 November 2018

melanocytic-differentiation antigens (MDAs), in normal and malignant melanocytes [6], is highly immunogenic and provokes immune responses against melanocytes, which sometimes lead to spontaneous disease regression. Immunological destruction of malignant melanocytes may cause hypopigmentation in a percentage of melanoma patients and is interpreted as favorable clinical sign of regression [7]. Nevertheless, there are hints that depigmentation is not in all cases a sign of melanocyte destruction by tyrosinase-boosted anti-melanoma cascades. Instead, depigmentation may indicate acquisition of invasive features [8–10]. An inverse correlation between the expression of tyrosinase and other MDAs and epithelial-mesenchymal transition (EMT) markers has been observed, where cells with low MDA levels demonstrate a mesenchymal-like phenotype and epithelial-like cells have high MDAs [11]. This leads to the hypothesis that EMT activation during melanoma progression might be facilitated by loss of expression and/or activity of MDA(s), however, comprehensive evidence is missing.

Melanoma progression is causatively associated with alterations in the TP73 gene, a functional and structural homologue of the p53 tumor suppressor [12,13]. TP73 synthesizes the transactivation-competent, anti-oncogenic TAp73 isoforms which derive from an external (P1) promoter; and the oncogenic DNp73 isoforms, which lack an intact transactivation domain. DNp73 further encompass the ΔNp73 isoforms arising from an internal (P2) promoter, and the ΔTAp73 isoforms derived from P1 after aberrant splicing in the N terminus [14,15]. ΔN's are also detected in normal tissues, while the ΔTA's are expressed exclusively in tumors and consistently predict poor patient outcomes [16]. We and others have demonstrated that the expression pattern of p73 isoforms is altered in melanoma, favoring upregulation of a specific splice-variant of the ΔTA class, namely p73ΔEx2/3 (hereafter also called DNp73) [17,18].

We have previously documented that DNp73 (p73ΔEx2/3) overexpression is a key event of melanoma progression, which independently from early driver mutations induces a mesenchymal phenotype switch via EMT activation [12], as well as pluripotency and stemness-like characteristics [13]. In the context of these studies, we further observed that when DNp73-overexpressing melanoma cells were injected into mice, they developed depigmented tumors. This coexistence of aggressive tumor features together with depigmentation in the same tumor intringued us to address pigmentation relative to invasion and shed light on a potential connection between EMT and tyrosinase in the context of aggressive melanomas. Herein, we show that DNp73 (p73ΔEx2/3) enhances tyrosinase ER-arrest and post-translational degradation in an IGF1R-dependent manner. Loss of active tyrosinase supports EMT, leading to depigmented, invasive and melanoblast-like cells.

2. Materials and methods

2.1. Cell culture and treatments

Human melanoma cell lines were maintained and treated as described in Supplemental Experimental Procedures.

2.2. Plasmid constructs, transfections, adeno- and lentiviral vectors

Plasmid constructs and viral vectors of this study are described in Supplemental Experimental Procedures.

2.3. Melanin assay and tyrosinase activity measurement

Melanin assay and tyrosinase activity measurement were performed as described in Supplemental Experimental Procedures.

2.4. Endo-H assay

Cell extracts were digested with 40 μg of endoglycosidase H (New England Biolabs) for 3 h at 37 °C, mixed with SDS sample buffer (Pierce) supplemented with 10% (v/v) 2-mercaptoethanol, and boiled at 95 °C for 5min. Samples were subjected to immunoblotting using the tyrosinase antibody.

2.5. Wound healing, matrigel invasion, proliferation assays, immunoblotting, immunofluorescence, ELISA and immunoprecipitation

Experiments were performed as described in the Supplemental Experimental Procedures.

2.6. RNA isolation and RT-PCR

RNA isolation and cDNA generation from cell lines and semi-quantitative reverse-transcriptase PCR has been described previously [13] using specific primer sequences (available upon request).

2.7. Microarray data

Microarray data were obtained and analyzed as previously described [12].

2.8. Xenopus embryo handling and whole mount RNA in situ hybridization

Experimentation on *Xenopus laevis* eggs was performed as described in Supplemental Experimental Procedures.

2.9. Mouse experiments and immunohistochemistry

Mice xenografts were produced and immunohistochemistry was performed on slices, as described previously [12] and in Supplemental Experimental Procedures.

2.10. Ethics statement

Experimental use of *Xenopus* embryos is licensed by the Government of Oberbayern (Projekt/AK ROB: 55.2.1.54-2532.6-3-11). Mouse experiments adhere to the Protocol on the Protection and Welfare of Animals and are approved by the local Animal Care Authorities.

3. Results

3.1. DNp73 (p73ΔEx2/3) promotes melanoma hypopigmentation via tyrosinase downregulation

We have previously shown that xenografts from less-invasive SK-Mel-29 melanoma cells that stably express DNp73 (SK-Mel-29.DNp73) develop aggressive characteristics through activation of IGF1R-mediated EMT cascades [12]. In continuation of that study, we intriguingly observed that these xenografts are hypopigmented compared to their mock controls (Fig. 1A). Consistently, SK-Mel-29.DNp73 cells show lower melanin synthesis rates than parental cells (Fig. 1B). These cells exhibit an inability for melanin synthesis, since supplementation of the cell culture media with increasing concentrations of the tyrosinase enzymatic substrate, L-tyrosine, triggers melanin production in SK-Mel-29, but not in SK-Mel29.DNp73 (Fig. 1C). Since tyrosinase is the rate-limiting catalyst of melanin synthesis, we investigated whether the inability of DNp73-expressing cells to convert L-tyrosine to melanin is attributed to changes in their tyrosinase status. Indeed, SK-Mel-29.DNp73-derived tumor xenografts exhibited decreased tyrosinase immunostaining relative to the SK-Mel-29 tumor sections (Fig. 1D). Consistently, lower tyrosinase protein levels were detected in DNp73-expressing melanoma cells accompanied by reduced enzymatic activity (Fig. 1E). This inverse correlation between tyrosinase and DNp73 was also found in other melanoma cell lines differing in their invasive/metastatic potential [12]. As demonstrated in Fig. 1F, all highly-

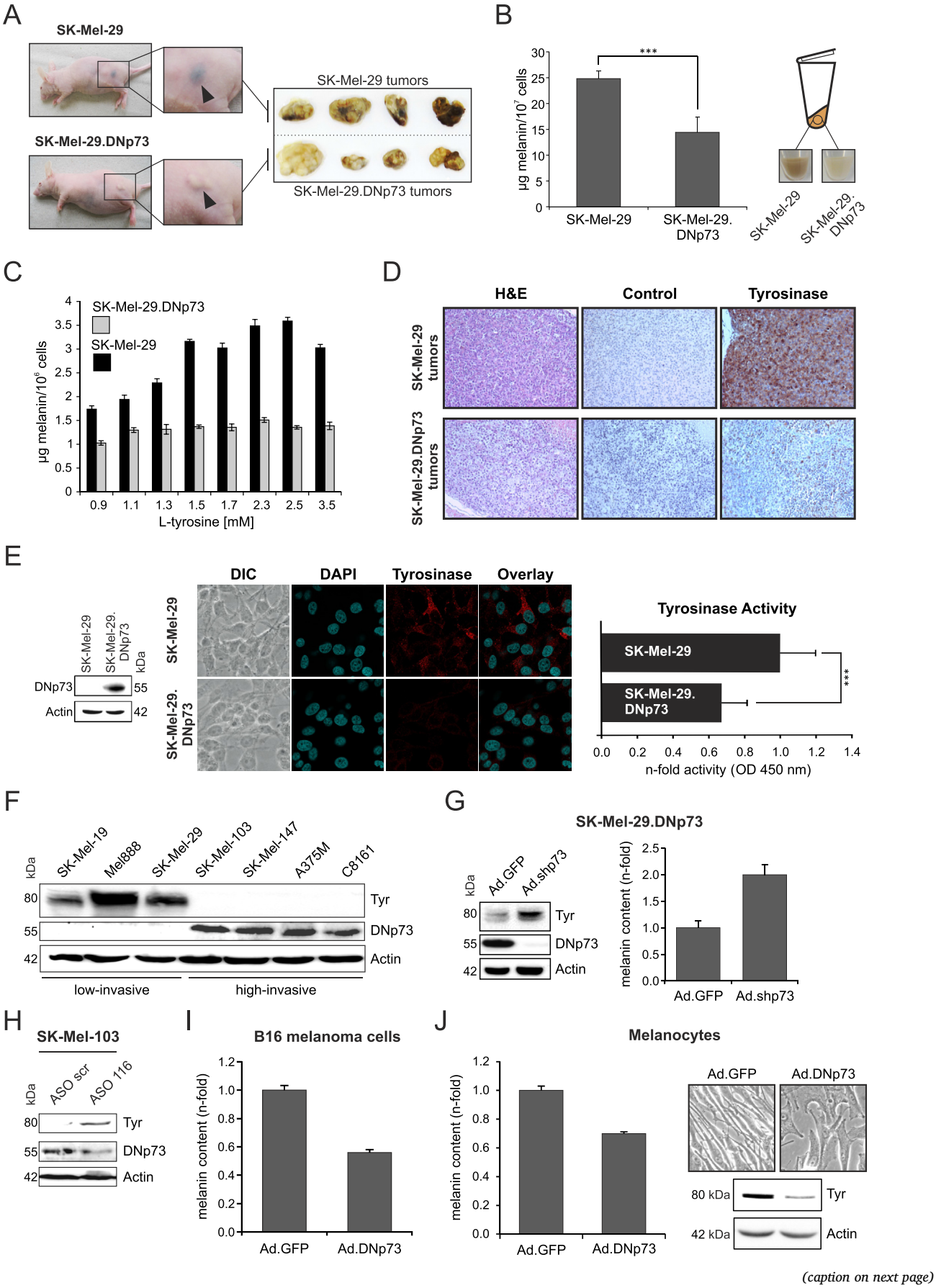

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Fig. 1. DNp73 (p73ΔEx2/3) induces tyrosinase loss and hypopigmentation. (A) Photographs of nude mice bearing SK-Mel-29 or SK-Mel-29.DNp73-derived melanomas and respective magnifications thereof showing pigment status of subcutaneously growing tumors (left panel). Excised tumors show differences in pigmentation intensity (right panel). **(B)** Melanin content of SK-Mel-29 and SK-Mel-29.DNp73 cells. Right panel shows cell sediments in Eppendorf tubes. **(C)** Melanin measurement following SK-Mel-29 or SK-Mel-29.DNp73 cells exposure to increasing L-tyrosine concentrations. Melanin production was triggered in parental SK-Mel-29 (black bars), but not in SK-Mel-29.DNp73, which remained insensitive to high L-tyrosine doses (grey bars). **(D)** H&E and tyrosinase stained tumor sections of SK-Mel-29 and SK-Mel-29.DNp73-derived melanomas. **(E)** DNp73 immunoblots in SK-Mel-29 and SK-Mel-29.DNp73 (left panel). Tyrosinase (red) and DNA (DAPI/turquoise) immunofluorescence of SK-Mel-29.DNp73 and SK-Mel-29 cells (center panel) with tyrosinase enzymatic activity in indicated cell lines (right panel). **(F)** Tyrosinase (Tyr) and DNp73 levels in low (SK-Mel-19, Mel888, SK-Mel-29) and highly invasive (SK-Mel-103, SK-Mel-147, A375 M, C8161) melanoma cell lines. Actin was used for equal loading. **(G)** Tyrosinase and DNp73 protein levels and melanin content after DNp73 knockdown in SK-Mel29.DNp73 transduced with Ad.shp73. **(H)** Immunoblots of DNp73 and tyrosinase upon inhibition of high endogenous levels of DNp73 using ASO116 in SK-Mel-103 cells. Protein levels were compared to cells treated with control ASOscr. **(I)** DNp73 overexpression in melanotic mouse B16 melanoma cells and through Ad infection, and subsequent melanin measurement. **(J)** Effect of ectopic DNp73 on melanin content in primary human melanocytes (left panel). Changes in cell morphology visualized by phase contrast microscopy and tyrosinase expression shown by Western blotting (right panel). Bar graphs represent mean ± SD from three independent experiments. $^{***}p < 0.001$ (Student's *t*-test, two-sided).

invasive tumor cells displayed strong endogenous DNp73 expression in conjunction with tyrosinase loss, while less-invasive cell lines showed low DNp73/high tyrosinase protein ratio. The effect of DNp73 on tyrosinase is also evident upon DNp73 silencing by anti-p73 shRNAs, which rescues tyrosinase loss and melanin synthesis in SK-Mel-29.DNp73 (Fig. 1G) as well as the p73ΔEx2/3 isoform-specific inhibitor ASO116 [12,19] in SK-Mel-103 with endogenously high DNp73, leading to tyrosinase increase (Fig. 1H). Notably, DNp73-mediated inhibition of melanin synthesis is functionally conserved between human and rodent cells, as evidenced by impaired pigment production in murine melanotic B16 cells upon transient DNp73 overexpression (Fig. 1I). These changes were also seen in primary, differentiated melanocytes that showed decreased pigmentation, diminished tyrosinase protein levels and a de-differentiated, 'melanoblast-like' morphology after transduction with DNp73 cDNA (Fig. 1J). Collectively, the DNp73 variant causes hypopigmentation through decreasing tyrosinase protein levels.

3.2. DNp73 (p73ΔEx2/3) induces ER arrest and proteasomal degradation of tyrosinase

Tyrosinase is regulated through transcriptional and post-translational mechanisms [20]. SK-Mel-29.DNp73 cells showed no alterations in *tyrosinase* mRNA levels relative to SK-Mel-29 (Fig. 2A). This was surprising, given that *TYR* promoter harbors p53-responsive elements which may bind p53 or TAp73 [21]. However, ectopic expression of p53, TAp73 or DNp73 in either SK-Mel-29 or SK-Mel-19 did not significally altered *tyrosinase* mRNA (Fig. 2B, top). Tyrosinase protein levels were drastically reduced by DNp73, but not by p53 or TAp73 (Fig. 2B, bottom). These results demonstrate that tyrosinase reduction is DNp73 isoform-specific and occurs regardless of the p53 binding site, in a transcription-independent manner, suggesting impairment of post-translational mechanisms of tyrosinase processing and/or stability.

Halaban et al. reported that in normal melanocytes, a portion of newly synthesized tyrosinase is diverted from the ER into degradation by the proteasome complex and that in some melanoma cell lines this process is accelerated. Proteasome inhibitors, such as MG132, can restore tyrosinase processing [3,5]. To evaluate if decreased tyrosinase levels are attributed to DNp73 interference with tyrosinase processing, we first applied an Endo-H assay on SK-Mel-29 in the presence or absence of DNp73, followed by tyrosinase Western blot. SK-Mel-29.DNp73 enriched the Endo-H-sensitive form (ca. 60-kDa), while SK-Mel-29 cells still show a strong signal of the Endo-H-resistant form at 80-kDa (Fig. 2C). This is accompanied by enhanced tyrosinase proteosomal degradation, as demonstrated by tyrosinase immunoprecipitation with an anti-Ub antibody, revealing an enhancement of ubiquitinated tyrosinase fractions in DNp73-expressing SK-Mel-29 cells versus parental cells (Fig. 2D). Treatment with MG132 increased the levels of processed tyrosinase and this was stronger in SK-Mel-29.DNp73 than in SK-Mel-29 (Fig. 2E). In the relatively low concentration of MG132, both tyrosinase and p27 used as positive control, were protected against proteasomal degradation. Tyrosinase was particularly accumulated in high DNp73 melanoma cells, indicating that the Endo-H-sensitive form of tyrosinase contained in these cells can be rescued from degradation even at low concentrations of proteasome inhibitor. Levels of the Endo-H-resistant form were also rescued. Our results are in agreement with earlier studies [5,22]. Tyrosinase protein folding in the ER is influenced by heat shock protein 70 (HSP70) family members [23] and ER-resident chaperones calnexin and calreticulin [24]. Thus, we examined if these genes are differentially expressed by analyzing microarray data from SK-Mel-29 and SK-Mel-29.DNp73. The heat map in Fig. 2F (left) revealed an upregulation of HSP70 transcripts and the ER chaperone calnexin in SK-Mel-29.DNp73. Interestingly, the ER-localized HSP70 family member HSPA5 (Bip/GRP78), which binds and retains tyrosinase in the ER [24], was verified to be significantly upregulated in DNp73-expressing cells (Fig. 2F, right). Further, bioinformatic analyses of patient data showed an increase of *HSPA5* in progressing melanoma (Fig. 2G), underlining a potential involvement of this chaperone in skin cancer development. Therefore, DNp73 might potentiate tyrosinase ER-retention and subsequent proteosomal degradation by upregulating HSP like HSPA5. In support, it was shown in an earlier work that HSP70 is also upregulated by p53 mutants [25], suggesting a similar mechanism for DNp73.

3.3. DNp73 (p73ΔEx2/3)-dependent tyrosinase loss is rescued by inhibition of the IGF1R pathway

To decipher which pathway links DNp73 with ER-arrest and tyrosinase proteosomal degradation, we took into account that HSPA5 is a novel downstream target of IGF1R/PI3K/AKT-mediated signaling [26]. PI3K/AKT signaling induces tyrosinase proteosomal degradation [27]. This leads to the hypothesis that HSPA5 increase followed by potentiation of ER-arrest and degradation could be due to an overactive IGF1R/AKT pathway which, as we have previously demonstrated, is stimulated in a high DNp73 melanoma cell context through the cross-talk of DNp73 with IGF1 receptor [12]. Indeed, inhibition of the IGF1R/PI3K/AKT axis via treatment of SK-Mel-29.DNp73 cells with either p-IGF1R inhibitor II (IGF1Ri) (Fig. 3A), PI3K inhibitor (Fig. 3B) or AKT inhibitor (Fig. 3C), resulted in AKT dephosphorylation followed by tyrosinase upregulation (Fig. 3A–C). This axis was substantially more enhanced than in low DNp73-expressing parental SK-Mel-29 cells (Fig. 3B–C), underscoring a link between DNp73-dependent tyrosinase loss and IGF1R pathway activity.

3.4. Tyrosinase loss enhances invasive capacity of melanoma cells

IGF1R/AKT cascade activation by DNp73 has been shown to induce enhanced cell motility [12]. Furthermore, with respect to the low DNp73/high tyrosinase protein ratio in non-invasive and inversed pattern in invasive melanoma cells (Fig. 1F), and the inhibition of the IGF1R pathway rescued tyrosinase expression (Fig. 3A–C), we investigated if tyrosinase changes affect invasiveness. Forced re-

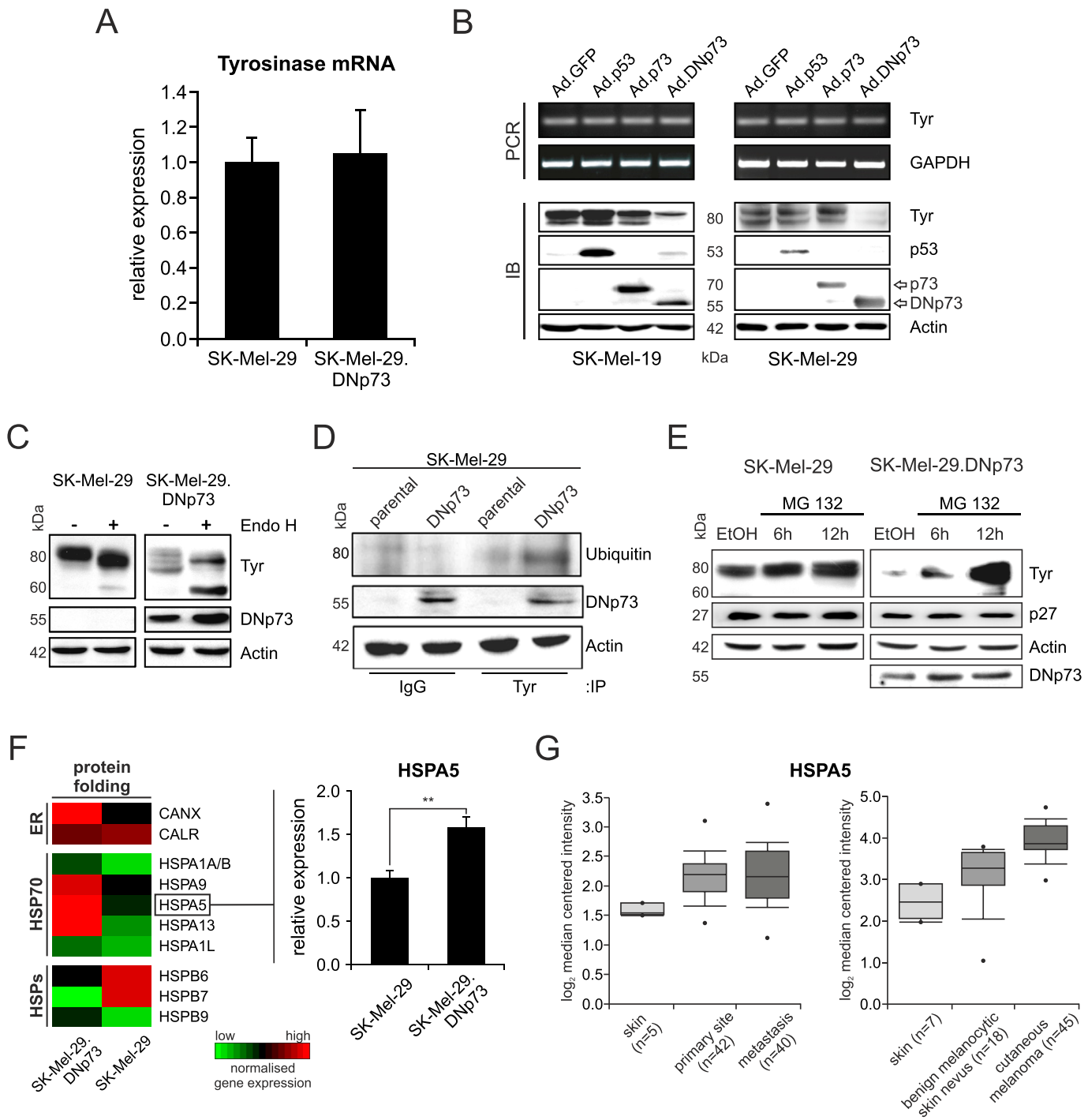

Fig. 2. DNp73 (p73ΔEx2/3) promotes ER-arrest and proteasomal degradation of tyrosinase, without affecting *TYR* transcription. (A) Quantification of *tyrosinase* mRNA levels in SK-Mel-29 and SK-Mel-29.DNp73 cells **(B)** RT-PCR (top) of tyrosinase expression in SK-Mel-19 and SK-Mel-29 cells transduced with Ad.p53, Ad.TAp73 or Ad.DNp73 vectors. Ad.GFP was applied as transduction control and GAPDH was used for equal loading. Expression of p53, TAp73 and DNp73 proteins after transductions was confirmed by immunoblots using anti-p53 and pan anti-p73 antibodies (bottom). Actin was used as a loading control. **(C)** Detection of tyrosinase protein in SK-Mel-29 or SK-Mel-29.DNp73 cell lysates with (+) or without (−) glycosidase digestion. Digestion of lysates from DNp73-expressing cells generates a band at 60-kDa representing ER-arrested, Endo-H-sensitive tyrosinase. Lysates from parental SK-Mel-29 cells are Endo-H-resistant, indicated by the appearance of a single band at 80-kDa, representing mature tyrosinase, which is glycosylated with more complex oligosaccharides. **(D)** Western blot of ubiquitinated tyrosinase protein eluates after immunoprecipitation with anti-tyrosinase antibody in DNp73-expressing and parental SK-Mel-29 cells. **(E)** SK-Mel-29 or SK-Mel-29.DNp73 cells treated with MG132 or EtOH as control. Protein lysates were harvested at indicated time points and tyrosinase was detected by immunoblotting. The MG132 concentration used was sufficient for inhibiting proteasomal degradation indicated by stable p27 levels in both cell lines at 6 and 12 h compared to the control. **(F)** Gene expression levels of several chaperons and heat shock proteins (HSP) from microarray data of SK-Mel-29 and SK-Mel-29.DNp73 (left panel). *HSPA5* expression was validated with qPCR in both cell lines (right panel). **(G)** Bioinformatic analysis of *HSPA5* expression in two different melanoma data sets (GSE7553 [55], GSE3189 [56]) from Oncomine database (www.oncomine.org). Boxes show the 25th to 75th percentile range of *HSPA5* levels. The solid lines within the boxes represent the median value and bars the 95% confidence interval. Circles are outliers. Data from three independent experiments are expressed as mean ± SD. $^{**}p < 0.01$ (Student's *t*-test, two-sided).

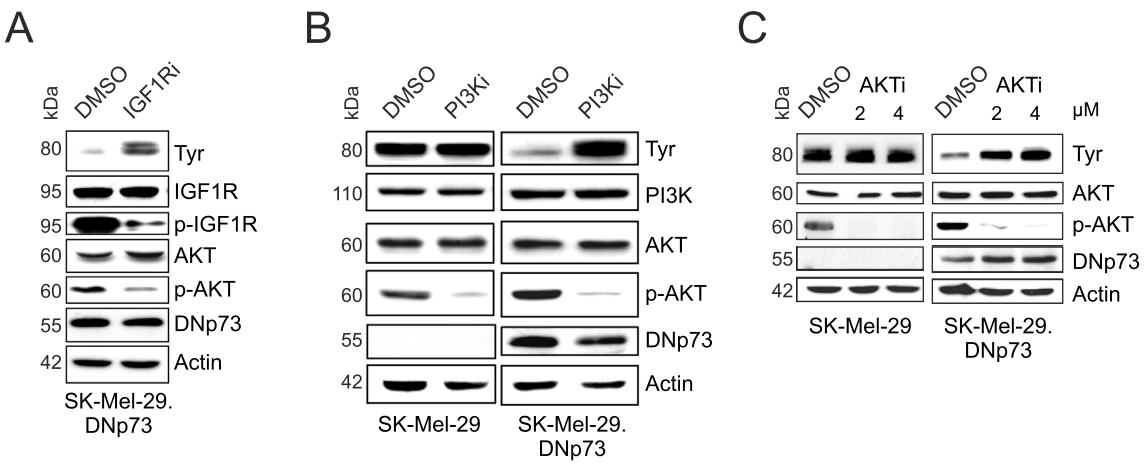

Fig. 3. DNp73 (p73ΔEx2/3) induced loss of tyrosinase is IGF1R-dependent. (A) SK-Mel-29.DNp73 treatment with 30 μM IGF1Ri for 1 h and subsequent Western blot analysis of DNp73, tyrosinase, total and phospho-IGF1R, as well as total and phospho-AKT levels compared to their DMSO-treated controls. **(B)** Immunoblots of tyrosinase, PI3K, AKT, p-AKT and DNp73 in the absence (DMSO) or presence of 20 μM of the PI3K inhibitor LY294002 (PI3Ki). **(C)** SK-Mel-29.DNp73 treated with increasing concentrations of AKT inhibitor VIII (AKTi) for 48 h DNp73, AKT, p-AKT and tyrosinase protein levels are indicated. Parental SK-Mel-29 cells were used as control for tyrosinase levels upon PI3K (B) or AKT (C) inhibitor treatment in the presence of low DNp73.

expression of tyrosinase in SK-Mel-147 or SK-Mel-103 remarkably reduced their invasive and migratory capacity and altered cell morphology (Fig. 4A–C). Reduced cell migration upon tyrosinase re-expression was again verified in other aggressive amelanotic cell lines such as A375 M, C8161, and WM793 (Fig. 4D), revealing that this effect is cell context-independent. Similarly, tyrosinase rescue in SK-Mel-29.DNp73 cells through transfection with pTyr abolished DNp73-induced melanoma invasion, but did not alter the motility in the endogenously tyrosinase expressing SK-Mel-29 (Fig. 4E). Vice-versa tyrosinase knockdown in melanotic SK-Mel-29 not only reduced melanin synthesis (Fig. 4F) but was also accompanied by enhanced cell invasion and migration (Fig. 4G). This was again observed in less-invasive SK-Mel-19 cells (Fig. 4G). Importantly, tyrosinase ablation in melanotic cells does not induce cell proliferation, which shows a specificity of tyrosinase for preventing cell invasion (Fig. 4H).

3.5. Tyrosinase loss induces EMT through altering the melanocyte ROS pool

In view of acquisition of an invasive and mesenchymal-like phenotype following tyrosinase knockdown, we examined whether this phenotypic switch is attributed to tyrosinase loss-induced alterations of EMT markers. Immunoblotting in less-invasive epithelial-like melanoma cell lines, in which endogenous tyrosinase is depleted through stable shTyr expression, demonstrated an EMT-phenotype with decreased E-cadherin and increased fibronectin, N-cadherin, vimentin, and Slug levels compared to sh.controls (Fig. 4I). Vice-versa, tyrosinase re-expression in mesenchymal-like, highly-invasive cancer cells, such as SK-Mel-147 and WM793, facilitated mesenchymal-epithelial transition, as evidenced by lowered expression of mesenchymal proteins (Fig. 4J) and diminished migratory capabilities (Fig. 4C and D).

Since tyrosinase is a melanosome-restricted oxidase, while Slug is a nuclear transcription factor and has never been reported as an oxidase substrate, a physical interaction between them is highly unlikely. To further elucidate how tyrosinase impacts EMT programs, we took into account its enzymatic nature. Tyrosinase decreases ROS burden of cells, because it converts melanin precursors that are essentially reactive oxygen species [2]. Since ROS induce EMT [28] and are etiologically linked with melanoma initiation and progression [29], we hypothesized that decreased levels of active enzyme might not suffice to scavenge ROS, subsequently leading to EMT activation. ELISA assays on SK-Mel-29 cells versus SK-Mel-29.DNp73 and SK-Mel-29.shTyr cells showed that ROS extracellular concentration increased in SK-Mel-29 cells upon either DNp73 overexpression or tyrosinase downregulation (Fig. 4K), indicating that DNp73-mediated depletion of tyrosinase leads to ROS accumulation, which can trigger EMT.

3.6. DNp73 (p73ΔEx2/3)-triggered link between depigmentation and EMT-dependent cell migration is conserved within the melanocytic lineage in Xenopus embryos

Melanocytes originate from a subpopulation of neural crest (NC) cells called melanoblasts. During development, melanoblasts migrate through the embryo, and when reaching their final sites, they are terminally differentiated to melanocytes. Melanocyte differentiation is a multistep and hierarchical program, from NC induction through premigratory and migratory NC cells to differentiated melanocytes. The migratory NC cells are the physiological sites of execution of EMT programs [1]. During melanoma progression, malignant melanocytes acquire features of their NC-precursors, such as *slug* reactivation [30]. To decipher whether the observed association between depigmentation and EMT-dependent cell motility is an ancestral trait undergoing co-option towards melanoma progression, we examined if it also exists in a highly regulated developmental setting, while NC cells become committed to melanocytes, using *Xenopus laevis* embryos as in vivo model to monitor melanocyte differentiation. In this case, DNp73 interference would recapitulate the advanced cancer phenotype (depigmentation and deregulated cell migration) in the developing embryo. Like its mammalian ortholog, the *Xenopus p73* gene produces multiple mRNAs, whose distinctly different open reading frames encode human TA- and ΔN-equivalent protein isoforms that are expressed broadly in the early embryo, including NC (data not shown).

Unilateral injection with mRNAs for either human DNp73 or control eGFP created embryos that consist of a microinjected side and a control side, the former being unambiguously identified by fluorescence from coinjected Alexa488-dextran (Fig. 5A and B). Significant depigmentation of melanophores populating the trunk and the eye of developing embryos was observed within DNp73-injected sides, compared to uninjected control sides or GFP-injected sides (Fig. 5C). In addition, RNA in situ hybridization for *tyrosinase* mRNA on DNp73-injected embryos displayed normal *tyr* gene expression pattern, indicating proper location of melanocytes and non−NC−derived retinal pigmented epithelium (RPE) cells (Fig. 5D–F). The affected melanocytes derive from NC cells, which become migratory around neural tube closure (NF19-21), following *snai1*-and *snai2/slug*-dependent EMT activation [31]. Interestingly, in unilaterally-injected embryos after neural tube stage (NF25), *slug* mRNA levels increased with high penetrance within the NC

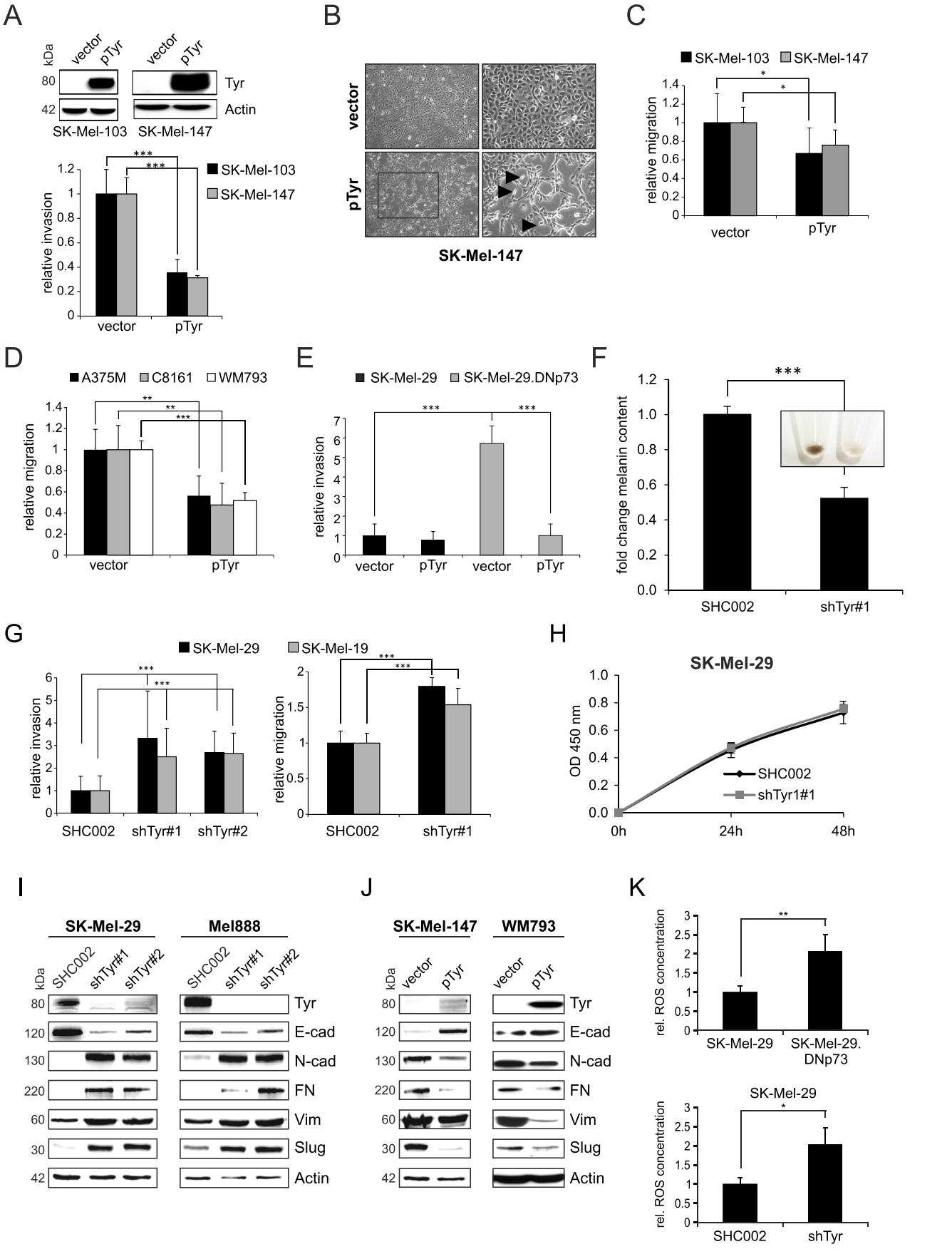

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Fig. 4. Tyrosinase suppresses melanoma cell motility and regulates EMT-related gene expression. (A) Tyrosinase expression in SK-Mel-103 or SK-Mel-147 cells 48 h post transfection with tyrosinase cDNA (pTyr) or empty vector (upper panel). Invasion assays on pTyr-expressing cells versus their mock counterparts appear in the lower panel. **(B)** Morphology changes of SK-Mel-147 overexpressing pTyr via phase contrast microscopy. SK-Mel-147 cells with a spindle-shaped invasive phenotype without dendritic processes appear with branched dendrites (arrows) under tyrosinase overexpression. **(C)** Wound healing assays showing relative migration of tyrosinase cDNA-expressing SK-Mel-147 and SK-Mel-103 cells versus controls. **(D)** Wound healing assays in indicated invasive melanoma cell lines. Fold changes are quantified 24 h after scratch, relative to mock controls. **(E)** Exogenous tyrosinase expression in SK-Mel-29 or SK-Mel-29.DNp73 cells and relative cell invasion 48 h post transfection. **(F)** Melanin assay of SK-Mel-29 cells stably expressing either a scrambled (SHC002) or anti-tyrosinase shRNA (shTyr#1). Diagram inlay shows sediments of respective clones. **(G)** Transfection of SK-Mel-29 and SK-Mel-19 cells with lentiviral shRNA.Tyr vectors. Boyden chamber and wound healing assays of SK-Mel-29 and SK-Mel-19 cells stably expressing shTyr#1, shTyr#2 or scrambled shRNA (SHC002) show increased invasion and migration upon shTyr-mediated knockdown. **(H)** XTT assay of SK-Mel-29.shTyr#1 versus SK-Mel-29.SHC002 at indicated times. **(I, J)** Western blot of tyrosinase (Tyr) and EMT markers E-cadherin (E-cad),N-cadherin (N-cad), fibronectin (FN), vimentin (Vim) and Slug in **(I)** SK-Mel-29 or Mel888 stably expressing shTyr#1, shTyr#2 or scrambled shRNA (SHC002) or in **(J)** SK-Mel-147 and WM793 cells transiently transfected with tyrosinase cDNA (pTyr) compared to controls (vector). Actin served as loading control. **(K)** Detection of ROS levels in SK-Mel-29.DNp73 versus SK-Mel-29 (top) and SK-Mel-29.shTyr#2 versus SK-Mel-29.SHC002 (bottom) at 24 h by ELISA. Bar graphs represent mean ± SD from three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student's *t*-test, two-sided).

(Fig. 6A, C), whereas *twist* mRNA expression remained unaltered (Fig. 6B, D). In analogy with this data, the DNp73/Tyr axis did not affect Twist1 levels in human melanoma cells (data not shown). Moreover, *slug* positive NC cells from the DNp73-injected side broadly initiate migration, compared to the uninjected side (compare inserts with close-ups of lateral views in Fig. 6A, bottom row). Thus, similar to invasive melanoma cells, DNp73 promotes depigmentation and migration in *Xenopus* NC cells via selective *snai2/slug* upregulation. Our findings indicate that DNp73 blocks melanin production by non-transcriptional regulation of tyrosinase in embryonic regions expressing *igf-1R*, i.e. in dorsoanterior tissues, including pigmented cells in eyes and trunk [32,33]. This is accompanied by changes in *slug* mRNA levels.

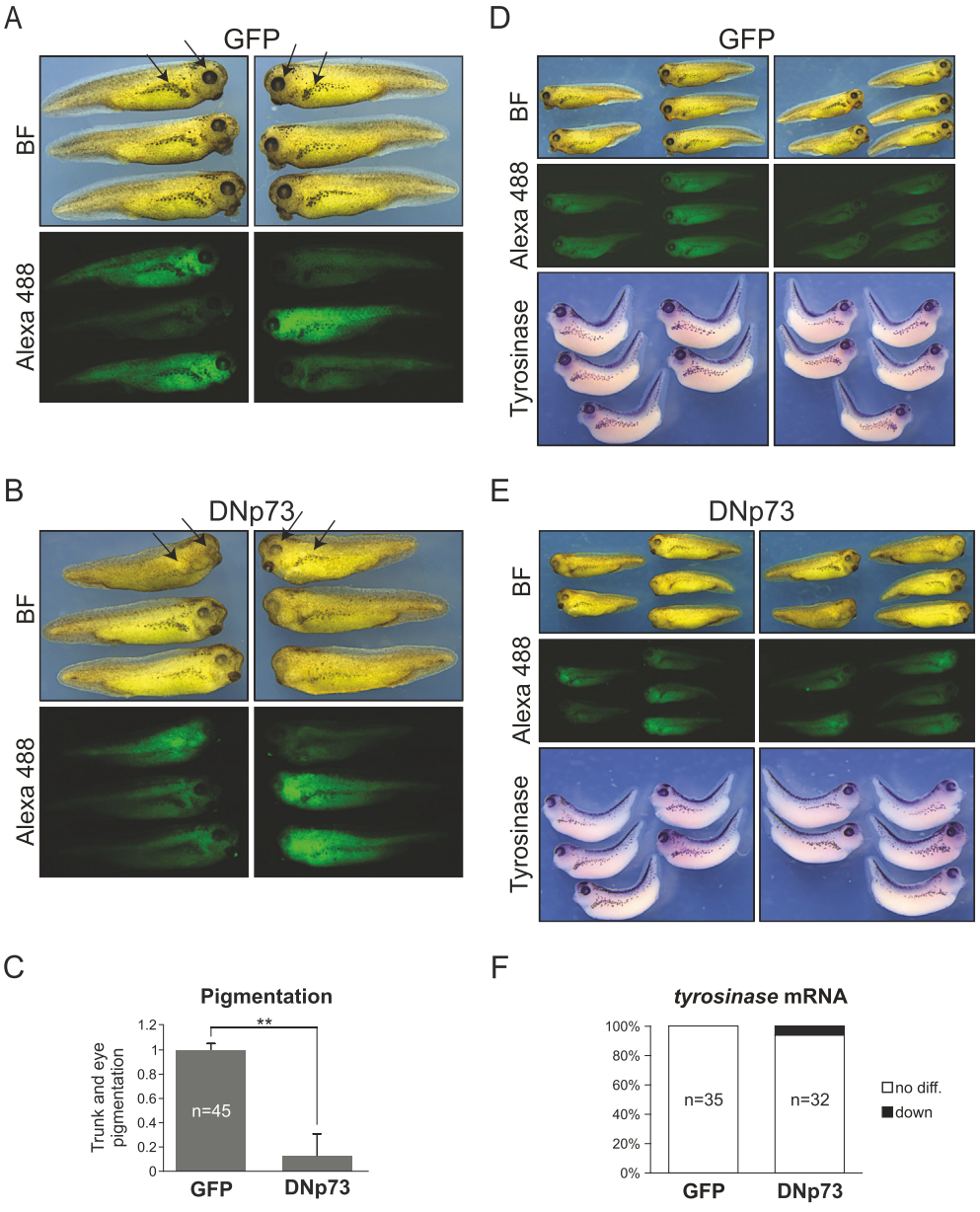

Fig. 5. DNp73 (p73ΔEx2/3) induces depigmentation without affecting *tyrosinase* transcription in *Xenopus* embryos. (A, B) Embryos injected with GFP **(A)** or DNp73 **(B)** mRNA plus Alexa488-dextran and subsequently fixed at NF36. The pCS2$^+$DNp73 and pCS2$^+$eGFP constructs were linearized using HpaI and NotI restriction enzymes and transcribed with SP6 polymerase. Synthetic mRNAs were injected at 0.2 fmol per blastomere of two-cell stage embryos (i.e. 500 pg of DNp73 and 120 pg eGFP mRNAs). One out of two blastomeres was injected ("unilateral injection"), resulting in embryos with a manipulated body half and an uninjected half, serving as internal control. Images depict both sides of three representative embryos per condition. Alexa488: fluorescent images of the same embryos on top. Black arrows mark darkly pigmented eyes and trunk melanocytes, respectively. **(C)** Frequencies of NF36 embryos with melanocyte hypopigmentation ($**p = 0.0087$; n = total number of embryos/condition; three independent experiments). **(D, E)** Embryos coinjected with Alexa488-dextran plus either GFP **(D)** or DNp73 mRNAs **(E)** were fixed at tadpole stage (NF36). Alexa488: lineage-tracing of injected sides. Melanin granules were bleached by H_2O_2 treatment and fixed embryos were subjected to RNA in situ hybridization for endogenous *tyr* mRNA. Importantly, note that: i) the same embryos shown in BF/Alexa488 images were batch-processed through RNA in situ hybridization with an anti-sense probe for *Xenopus tyrosinase* mRNA; the involved dehydration step ii) inactivates Alexa488 fluorescence and iii) causes the U-bent embryo shape. None of the five embryos, displaying unilateral hypopigmentation, shows comparable reduction in *tyr* mRNA expression. **(F)** Frequency of embryos with unilaterally reduced *tyr* mRNA levels (data from three independent experiments). BF: brightfield image.

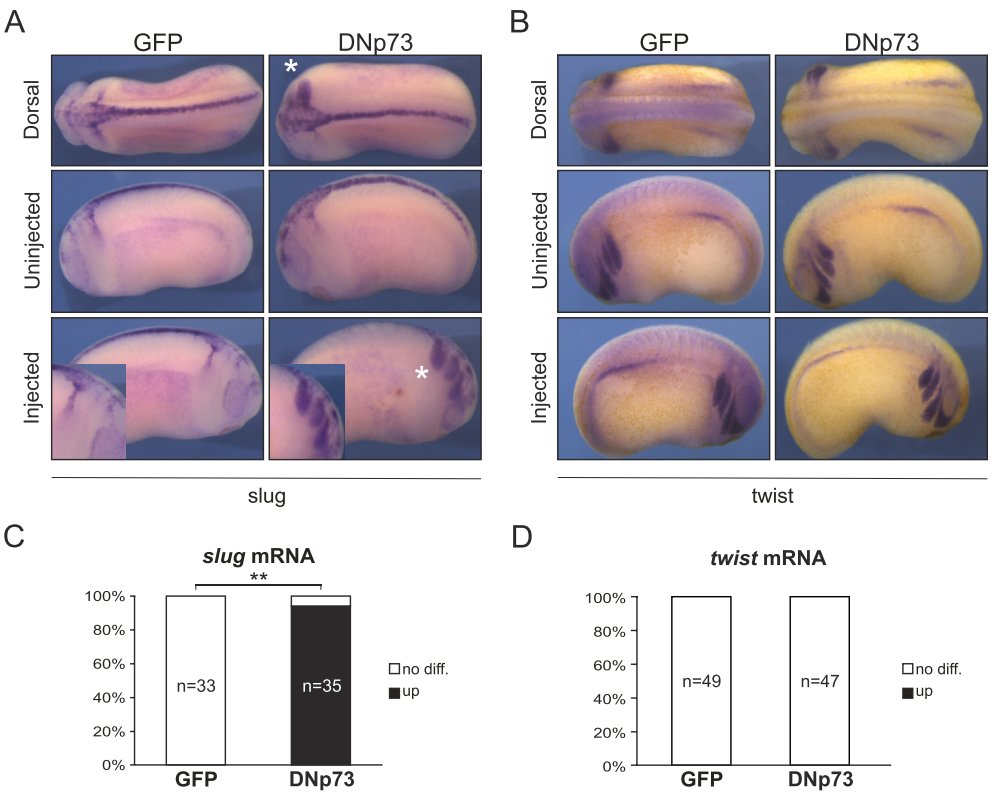

Fig. 6. DNp73 (p73ΔEx2/3) superinduces *slug* transcription in *Xenopus*. **(A, B)** Embryos injected unilaterally with eGFP or DNp73 mRNAs were fixed at early tail bud stage (NF24-25), sorted under fluorescent light from coinjected Alexa488-dextran into left- and right-side injected embryo cohorts for both conditions, and subjected to RNA in situ hybridization for *Xenopus slug* **(A)** or *twist* **(B)** expression. Two representative, right side-injected embryos injected are shown under different angles of view, as indicated on the left side. White asterisks denote superinduction of *slug* mRNA within migratory NC on the DNp73-injected side. **(C, D)** Frequencies of embryos with symmetric or unilaterally upregulated mRNA expression. *twi*: no difference, four independent experiments; *slug*: majority shows upregulation, three independent experiments (**$p = 0.0013$).

4. Discussion

Immune responses against malignant melanocytes, either spontaneous or after immunotherapy, may lead to melanoma-associated hypopigmentation (MAH) in a subpopulation of melanoma patients. Although MAH tends to be interpreted as favorable clinical sign of regression [7], there is an increasing number of enigmatic case reports of patients with MAH who experienced disease progression [34–39]. Additionally, metastatic melanoma biopsies with decreased tyrosinase staining [40] and undifferentiated/dedifferentiated cases of metastatic melanoma negative for tyrosinase were observed [41]. These clinical results warrant revisiting in the era of precision medicine, especially in light of the fact that depigmentation is a reported side-effect of promising immunotherapy formulations [42,43]. They timidly counterpropose that the empirical rule-of-thumb of skin hypopigmentation as a good prognostic factor might not be universally applicable and that complementary molecular markers are needed to decrease the risk for underrecognition and/or misdiagnosis. MAH emerges as an ambiguous clinical sign, suggesting either melanocyte destruction by anti-melanoma cascades or appearance of colorless melanoblast-like variants with enhanced invasive capacity. However, lack of accurate molecular aids poses as a bottleneck of accurate diagnosis of depigmented melanomas.

Herein, we show that in aggressive melanoma tumors with overexpression of p73ΔEx2/3, the tumor-specific, aberrantly splice-variant of the TP73 gene, depigmentation occurs. Melanin reduction is attributed to ER-retention and proteosomal degradation of tyrosinase as a consequence of an DNp73-upregulated IGF1R/PI3/AKT pathway. Tyrosinase loss, in turn, triggers EMT markers' expression, mesenchymal-like cell phenotypes and increased cell migration and invasiveness. The activation of a DNp73/IGF1R axis in combination with acquisition of mesenchymal cell characteristics could be considered as a molecular diagnostic means to discriminate if a MAH indicates regression or progression.

The ability of the p73ΔEx2/3 variant to induce depigmentation is conserved in lower vertebrates. DNp73-induced depigmentation coupled with Slug overexpression and altered cell migration is recapitulated in *igf-1R*-positive areas of *Xenopus* embryos, i.e. in dorsoanterior tissues, including pigmented cells in eyes and trunk [32,33]. In contrast, in non-*igf-1R*-expressing cells (ventral midline), DNp73 elicits cell proliferation. This indicates that cells are susceptible to DNp73 insults but get committed to distinct fates reminiscent of either melanoma initiation (proliferation) or progression (depigmentation and impairment of cell migration), depending on their IGF1R content. Furthermore, it suggests that a physiological association between tyrosinase expression and Slug inhibition exists in normal cells of NC-origin, which is perturbed by cancer-specific DNp73. DNp73 blocks melanin production via non-transcriptional deregulation of tyrosinase in embryonic regions expressing *igf-1R* [32,33] and affects migration through selective *slug* inhibition, leaving *twist* unaffected.

Tyrosinase transcription can be regulated by p53 family members [44]. In particular, p53 or TAp73 may bind to p53 binding sites of the *TYR* promoter [21], while p53 is both a positive [44] and a negative [45] transcriptional regulator of tyrosinase, depending on the melanoma cell context. Note withstanding, the cancer-specific p73ΔEx2/3 acts downstream of tyrosinase transcription, at the post-translational level, bypassing the well-described mechanisms of dominant-negative inhibition of the anti-oncogenic p53 or TAp73 [16]. Moreover, given that upon NRAS/BRAF activation, EMT-TF network is reorganized in favor of Twist [46], it is obvious that DNp73 has the ability to induce EMT-TF reprogramming on its own right, independent of networks governed by early driver mutations. This data collectively demonstrates that this particular DNp73 isoform may invent an own 'repertoire' of invasive pathways, in compliance with the recent finding that cancer-specific protein variants can exhibit neomorphic-type activities and develop interactions which do not naturally occur in normal cells [47]. By strategically destabilizing tyrosinase, DNp73 releases the break on EMT via perturbing the tyrosinase-Slug association which had been established early in melanocyte differentiation. Thus, it facilitates re-acquisition of characteristics of melanoblast-like precursors, possibly 'undisturbed' by any antagonistic effects of co-expressed TAp73 tumor-suppressive antagonists and via overcoming the NRAS/BRAF/Twist

axis.

The aggressive features of tyrosinase-negative melanoma lesions can be comprehended if we view this antigenic type of tumor through an immunology lens. In particular, aggressive melanomas present intratumoral heterogeneity, consisting of subpopulations of cell variants, which offer phenotypical plasticity [9]. Under the selective pressure of increased immune surveillance, cell variants which can invade tissues undisturbed from immune responses are particularly favored. In this respect, low-MDA expressing cell variants are immunoselected [48], because they are poorly recognized by MDA-specific T-lymphocytes [11,49]. Therefore, cell variants which fail to produce tyrosinase protein become 'invisible' to the tyrosinase-targeting immune cells, even if their ability to synthesize *tyrosinase* mRNA is intact or enhanced. Overall, tyrosinase loss can contribute to both, immune-escape [48] and invasion. This simultaneous acquisition of two cancer hallmarks [50] theoretically offers depigmented cells a dual selective advantage over the pigmented, tyrosinase protein-producing variants, which are less invasive and 'visible' by the immune system. A model of melanoma progression by Hoek and colleagues suggests that invasion and proliferation are interchangeable transcriptional programs between which melanoma cells oscillate during progression in response to changing microenvironmental signals [7]. Our results reinforce this model and provide further hints that the switch between invasion and proliferation can be additionally achieved through post-transcriptional mechanisms, such as proteosomal degradation of tyrosinase. This oscillating pattern could, at least in part, reflect a 'hide and seek' scenario between the immunogenic melanoma tumor and the immune system, aiming at survival and rapid spread of the tumor. In this scenario, overproliferating, high tyrosinase-expressing melanocytes provoke anti-tyrosinase responses, which in turn add pressure for the emergence of dedifferentiated low tyrosinase-expressing melanoblast-like cell variants with active EMT programs. These variants then exit the primary tumor and migrate to distant sites, where they proliferate and become redifferentiated to tyrosinase-expressing cells, giving rise to a secondary pigmented tumor and so on.

Tyrosinase vaccines are being evaluated in clinical studies as melanoma-specific therapies [51]. In light of this, we should keep in mind that melanomas are dynamic and evolving structures and what is appropriate to manage early-stage tumors demonstrates poor efficiency when used to treat invasive stages. Tyrosinase vaccines [51] and inhibitors of melanoma-initiating gain-of-function BRAF/NRAS mutations constitute such examples [52]. Important is that subpopulations of drug-resistant cells survive acute treatment via engagement of an activated IGF1R/PI3K/AKT signaling cascade [53]. In this respect, given that a DNp73-IGF1R cross-talk master-regulates a series of molecular events that facilitate acquisition of dedifferentiation and stemness characteristics (ref. [12,13] and this study), it emerges as an appealing anti-invasive target. Given that tumor and immunity co-evolve in melanoma patients under immunotherapy [54], future investigation on how modulation of the DNp73/IGF1R-governed prometastatic network could restore immunogenicity and/or drug sensitivity might open new avenues for next-generation diagnostics and therapeutics.

Our results may hold a diagnostic promise for discriminating between patients with MAH-associated metastatic disease and those, where MAH is indeed a sign of regression. We show that in depigmented invasive melanomas, a DNp73-upregulated IGF1R/AKT pathway destabilizes tyrosinase by enhancing its ER-arrest and post-translational degradation. Tyrosinase loss in high DNp73-expressing melanomas enhances EMT activation and transformation of melanocytes to melanoblast-like cells, reminiscent of their Slug-positive migratory NC cell precursors. Hence, the DNp73/IGF1R/Slug molecular signature in colorless lesions might be proven useful to stratify the special subpopulation of melanoma patients presenting with MAH.

Funding

This work was partially supported by Rostock University Medical Center for the project Systems Medicine of Cancer Invasion and Metastasis and by grant 70112353 from the Deutsche Krebshilfe (German Cancer Aid) to B.M. Pützer and by a DFG grant in the framework of SFB1064 (project A12) to R.A.W. Rupp.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to Anja Stoll for technical assistance and to Professor Michael G. Sargent, MRC National Institute for Medical Research (UK) for providing Xenopus twist and slug expression plasmids.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.009.

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