A natural WNT signaling variant potently synergizes with *Cdkn2ab* loss in skin carcinogenesis

Paul Krimpenfort\(^1,2\), Margriet Snoek\(^2\), Jan-Paul Lambooij\(^1,2\), Ji-Ying Song\(^3\), Robin van der Weide\(^1,4\), Rajith Bhaskaran\(^1,2\), Hans Teunissen\(^1,4\), David J. Adams\(^5\), Elzo de Wit\(^1,4\) & Anton Berns\(^1,2\)

*Cdkn2ab* knockout mice, generated from 129P2 ES cells develop skin carcinomas. Here we show that the incidence of these carcinomas drops gradually in the course of backcrossing to the FVB/N background. Microsatellite analyses indicate that this cancer phenotype is linked to a 20 Mb region of 129P2 chromosome 15 harboring the *Wnt7b* gene, which is preferentially expressed from the 129P2 allele in skin carcinomas and derived cell lines. ChIPseq analysis shows enrichment of H3K27-Ac, a mark for active enhancers, in the 5' region of the *Wnt7b* 129P2 gene. The *Wnt7b* 129P2 allele appears sufficient to cause in vitro transformation of *Cdkn2ab*-deficient cell lines primarily through CDK6 activation. These results point to a critical role of the *Cdkn2ab* locus in keeping the oncogenic potential of physiological levels of WNT signaling in check and illustrate that GWAS-based searches for cancer predisposing allelic variants can be enhanced by including defined somatically acquired lesions as an additional input.

---

1 Oncode Institute, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. 2 Division of Molecular Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. 3 Department of Experimental Animal Pathology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. 4 Division of Gene Regulation, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. 5 Wellcome Trust Sanger Institute, Wellcome Genome Campus, Cambridge CB10 1SA, UK. Correspondence and requests for materials should be addressed to A.B. (email: a.berns@nki.nl)
The CDKN2ab locus on chromosome 9p21 in human (chromosome 4 in mouse) harbors the genes for three tumour suppressor proteins: p16INK4a and p14ARF (p19Arf in mice) encoded by CDKN2a and p15INK4b by CDKN2b (ref. 1). Both p15INK4b and p16INK4a are able to induce cell cycle arrest in G1 by inhibiting cyclin-dependent kinases CDK4 and CDK6 thereby relieving the cell cycle suppression through the retinoblastoma (RB1) family of tumour suppressor proteins. The unrelated p14ARF protein acts primarily through MDM2 to activate the key checkpoint protein TRP53, thereby inducing either cell cycle arrest (both in G1 and in G2) or apoptosis. The entire locus is frequently lost in a wide range of primary tumours.

Previously, we have described the generation Cdkn2ab knockout mice derived from 129P2 ES cell clones. The first generations of Cdkn2ab knockout mice on a mixed 129P2;FVB/N background frequently developed skin tumours and various soft tissue sarcomas, tumour types not normally seen in Cdkn2a−/− mice2. However, the skin tumour phenotype was gradually lost upon backcrossing the knockout Cdkn2ab allele onto the FVB/N background suggesting the involvement of one or more 129P2 alleles in addition to Cdkn2ab loss in skin tumour development.

In this study to gain insight into this phenomenon we repeated the procedure to generate Cdkn2ab knockout mice, but for the backcrossing to FVB/N we now selected skin tumour bearing mice to enable the identification of potentially relevant 129P2 loci involved by analysing the 129P2 contribution in the progeny with and without the skin tumour phenotype. Microsatellite analyses indicate that the skin cancer phenotype is linked to a 20 Mb region of 129P2 chromosome 15 harbouring the Wnt7b gene. The results show that the Cdkn2ab locus fulfils a critical role in keeping the oncogenic potential of physiological levels of WNT signalling in check. In addition, our study illustrates that the identification of cancer predisposing alleles in genome-wide association studies might be facilitated by focussing on tumours carrying distinct somatically-acquired oncogenic lesions3.

Results

Cdkn2ab knockout mice develop tumours. The search for the relevant 129P2 allele(s) involved in the skin cancer phenotype of Cdkn2ab KO mice was started by generating chimeric mice using the 129P2 ES cell clone carrying knockout alleles for p15Ink4b and p16Ink4a and conditionally knockout for p19Arf (ref. 2). This compound mutant allele, which we called Ink4ab−, enables Cre-mediated inactivation of p19Arf thereby generating a mutant Cdkn2ab locus for all three tumour suppressor proteins (Supplementary Figure 1). Chimeric mice generated from Ink4ab− ES cells were crossed to FVB/N mice harbouring a germline-expressing Cre transgene (Act-Cre) to obtain F1 129P2;FVB/N mice carrying either an Ink4ab− allele or a Cdkn2ab− allele. Subsequently, these mice were intercrossed to obtain a cohort of homozygous Cdkn2ab−/− mice unable to express any of these three tumour suppressor proteins, on a mixed 129P2;FVB/N background.

Within 4 months, many Cdkn2ab−/− animals develop multiple skin tumours in addition to other tumour types. Per animal up to 15 skin lesions develop synchronously all over the trunk (Fig. 1a) and are first detectable by palpation at the age of 8 weeks. The

Fig. 1 Cdkn2ab−/− mice show multiple skin trichoblastic carcinomas. a Total scan image of the skin region representing multiple skin tumours (arrows, scale bar 100 mm). b H&E and IHC characterization of skin tumours consisting largely of hair germ (LEF1+, P-CAD+, SOX9+), basal/squamous (CK14+/CK10+) cells and dermal papillary cells (LEF1+), also showing strong regional β-Catenin positivity (scale bars: 20 μm)
The Chr 15: 85 MB region synergises with Cdkn2ab loss. To identify the relevant 129P2 allele(s) in affected mice we performed a genome-wide scan using 134 microsatellite markers selected to be polymorphic between 129P2 and FVB/N with an average coverage of ~20 Mb (~10 cM) (for information on microsatellites see Supplementary Table 1). Of a group of 38 Cdkn2ab−/− mice of the first generations, 13 mice developed skin tumours with strong genetic linkage to a marker on Chromosome 15 (D15Mit107 at 85 Mb). Further backcrossing of skin tumour bearing Act-Cre;Cdkn2ab−/− mice to Ink4ab−/− mice (FVB/N background) supported the linkage to this chromosomal segment and subsequent fine mapping identifies an interval of 20 Mb on Chromosome 15 (Chr15: 67–87 Mb) (Fig. 3a) with genetic linkage to the skin carcinoma phenotype. No Cdkn2ab−/− mice homozygous for the FVB allele at Chr15: 85 Mb region developed skin carcinoma’s (n = 95), whereas the penetrance of the skin carcinoma phenotype in mice heterozygous for 129P2 allele at Chr15: 85 Mb is over 75% (70 out of 91) (Fig. 3b, c). Likewise, these data also support the contribution of Cdkn2ab loss and the 129P2 Chr15: 85 Mb region to aberrant squamous differentiation in the lung (Fig. 3d) since these lesions are observed almost exclusively in skin tumour bearing mice. In contrast, the other tumour types are observed in Cdkn2ab−/− mice with or without the 129P2 Chr 15: 85MB allele (Fig. 3e).

Mouse strains carrying Cdkn2a (ref. 8–10) or Cdkn2b (ref. 11) mutant alleles published previously do not develop skin tumours. However, the cohorts analysed in these studies did not carry the 129P2 Chr15: 85 Mb region. To determine whether the loss of all 3 tumour suppressors encoded by the Cdkn2ab locus is required for skin carcinogenesis we generated 3 additional cohorts of mice carrying the relevant 129P2 region but each heterozygous knockout for either one of the three Cdkn2ab encoded proteins while being full knockout for the other 2 (see Supplementary Figure 1 for schematics of all Cdkn2ab mutant alleles used in this study). Skin tumours develop in a small fraction (10%) of Cdkn2a knockout mice hemizygous for Cdkn2b, however, with a much longer latency than full Cdkn2ab knockout mice (Fig. 3e). No skin tumours are seen in mice heterozygous for modified alleles still encoding either p16Ink4a or p19Arf only. These data indicate that each of the three Cdkn2ab encoded proteins can effectively suppress skin carcinogenesis in mice carrying the 129P2 Chr15: 85 Mb region.

The Chr15: 67–87 Mb region harbours, among many other genes (see Supplementary Table 2), the genes encoding Wnt7b and Pdgfβ. Both genes are functional in putative oncogenic signalling pathways and fulfil a critical role in the development of hair follicles12,13 and lung14,15. In addition, p15Ink4b is highly expressed6,17 during the development of these tissues. Therefore, we decided to further explore the relevance of these genes in combination with the complete Cdkn2ab loss in skin carcinomas and lung lesions. mRNA

![Image](https://example.com/image1)

**Fig. 2** Cdkn2ab−/− mice show squamous lung metaplasia. H&E (scale bars 200 μm and 20 μm) and CK5, SOX2 and P63 IHC (scale bar: 20 μm).
Fig. 3 Identification of the Chr15 79–87 Mb region having genetic linkage to the skin tumour phenotype. 

**a** Schematic of the recombined Chr15 with varying 129P2 and FVB/N background in skin tumour bearing mice (blue: 129P2, green: FVB/N background and grey: region of crossover); relevant microsatellite markers used are indicated on top; below a blowup of the relevant region. 

**b** Number of skin tumours on individual Cdkn2ab−/− mice homogous FVB/N (green) Chr15 85 Mb or FVB/N;129P2 (blue) for region Chr15 85 Mb (Chr15*). 

**c** Tumour spectrum of Cdkn2ab−/− mice as mentioned in **b**. 

**d** Frequency of squamous metaplasia in the lungs of Cdkn2ab−/− mice as mentioned in **b**. 

**e** Kaplan–Meier curve showing skin tumour-free survival of Cdkn2ab mutant mice FVB/N;129P2 for the Chr 15; 85 Mb region; red line: Cdkn2ab−/−: deficient for p15Ink4b, p16Ink4a and p19Arf; green: Cdkn2b−/−;Arf+/−;Ink4a−/−: deficient for p15Ink4b and p16Ink4a, proficient for p19Arf; blue: Cdkn2b−/−;Arf−/−;Ink4a+/−: deficient for p15Ink4b and p19Arf, proficient for p16Ink4a; orange: Cdkn2b−/−; Cdkn2a−/−: deficient for p15Ink4b and p19Arf, proficient for p15Ink4b. Statistical analysis (Unpaired t-test): ns: ***p < 0.01, ****p < 0.001

---

Fig. 4 WNT7b and PDGFβ expression in skin tumours and lung metaplasias. 

**a** mRNA (qPCR) analysis of Wnt7b and Pdgfβ (relative to control) of 5 independent skin tumours (red triangle) and three soft tissue sarcomas (c). 

**b** Total scan image (scale bar: 100 mm), and **c** high power image of WNT7b IHC in primary skin tumours (scale bar: 20 μm). 

**d** High power image of WNT7b IHC in lung metaplasia (scale bar: 20 μm). Statistical analysis (Unpaired t-test): ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent standard deviation of the mean (3 replicates)
analysis and immunohistochemistry show that Wnt7b is expressed in the skin carcinomas of Cdkn2ab−/− mice (Fig. 4a–c) but not in soft tissue sarcomas. Wnt7b expression is also prominent in the squamous lesions in the lungs of these animals (Fig. 4d). Pdgfβ is expressed in the skin tumours as well, but also in the soft tissue sarcomas (Fig. 4a).

Allele-specific mRNA analyses based on 3′ UTR polymorphisms (see Methods section) shows that in the skin carcinomas both genes are transcribed primarily from the 129P2 alleles, explaining the dependence of skin carcinoma development on the presence of this allele (Fig. 5a). Notably, the Fibulin1 gene located in between the Pdgfβ and Wnt7b genes does not show allele-specific expression. Since the amino acid sequences of both PDGFB and WNT7b encoded by FVB/N and 129P2 genes are identical this suggests that variation in their regulatory sequences or post-transcriptional mRNA stability underlies the enhanced expression of the 129P2 alleles in the skin tumours. To assess whether the 129P2 region has an altered chromatin landscape, we derived tumour cell clones able to grow in soft agar from three independent skin tumours. In these clones Wnt7b expression is reduced and Pdgfβ expression is hardly detectable (Supplementary Figure 4a). However, as observed in the primary tumours, in these cell clones the Wnt7b gene is transcribed with a similar allele specificity although to a lesser extent (Supplementary Figure 4b). We performed H3K27-Ac ChIP-seq analysis on three clones derived from independent tumours to identify active enhancers. Of note, the cell lines analysed shows a gain of FVB/N chromosome 15, leading to an average allelic balance of 33 percent 129P2 instead of 50 percent. The allele-specific analysis identifies four regions with a significant increase (i.e. higher than the expected allelic balance) of H3K27-Ac on the 129P2 allele (Supplementary Figure 5). One of these regions, chr15:85643259–85648162, is juxtaposed upstream of a known super-enhancer mentioned in the dbSUPER database (https://www.ncbi.nlm.nih.gov/pubmed/26438538) (Fig. 5b). This region is located 52 kb upstream of the Wnt7b promoter and has a 2-fold higher fraction of the 129P2 allele (FDR < 1%). The relative increase of H3K27-Ac in this super-enhancer region on the 129P2 allele is consistent with the allele preference of Wnt7b transcriptional activity. There are multiple polymorphisms in this super-enhancer region also affecting several transcription factor binding motifs. Further investigation is needed to identify the relevant motifs responsible for the here observed differential transcriptional control of Wnt7b.

Transformation by Wnt signalling is mediated by Cdk6. To gain insight into the underlying mechanism of the cooperation between enhanced Wnt7b and Pdgfβ expression and loss of the Cdkn2ab locus we turned to defined cell culture systems: MEFs and tumour-derived cell lines. We used MEF’s from Cdkn2a−/− and Cdkn2ab−/− mice not carrying the 129P2 Chr15: 67–87 Mb region. Since Wnt7b acts in the canonical WNT signalling pathway, we used the well-defined WNT3a conditioned medium as a source of WNT protein to assay their responsiveness to WNT signalling. We transfected a WNT-responsive Luciferase reporter construct in Cdkn2a−/− and Cdkn2ab−/− MEF’s and show that β-catenin dependent transcription is strongly induced by WNT3a supplementation in Cdkn2a−/− and Cdkn2ab−/− MEF’s to comparable levels (Supplementary Figure 6a). A control Luciferase reporter construct not containing Wnt responsive elements is hardly expressed in Cdkn2a−/− and Cdkn2ab−/− MEF’s with or without WNT3a supplementation. However, Cdkn2ab−/− MEF’s shows a much stronger proliferative response to WNT stimulation than Cdkn2a−/− MEF’s in adherent cultures (Supplementary Figure 6b). In contrast, PDGFB does not affect proliferation of Cdkn2ab−/− MEF’s with or without WNT3a supplementation (Supplementary Figure 7). Using 10% serum HITES medium (see Methods) WNT supplementation also strongly promotes colony formation and growth in soft agar of Cdkn2ab−/− but not of Cdkn2a−/− MEF’s (Fig. 6a). These results indicate that, at least in MEFs, p15Ink4b restricts the effects of canonical WNT signalling.

Next, we turned to the cell clones derived from the Cdkn2ab−/− skin tumours and focused on unique features associated with transformation i.e., anchorage-independent growth and migratory capacity. Under regular adherent culture conditions supplying additional PDGFB does not change the proliferation of these tumour cell clones nor does it affect their anchorage-independent growth (Supplementary figure 8). WNT3a conditioned medium has no substantial effect on the growth of tumour cell clones under adherent conditions but...
significantly stimulates the proliferation in soft agar conditions although this response does vary between tumour cell clones (Supplementary figure 9 and Fig. 6b, c). These data indicate that WNT signalling is critical for the anchorage-independent colony formation of tumour cells and this raises the question to what extent the WNT ligands produced by the tumour cells themselves are determining their growth behaviour. To test this, we used the C59 Porcupine inhibitor to block WNT palmitoylation and thereby its function. Addition of C59 prevents their anchorage-independent growth, which can be rescued by WNT3a conditioned medium (Fig. 6b, c). However, since C59 affects the function of all WNTs this experiment did not show that the elevated expression of WNT7b is required for soft agar growth. Therefore, we disrupted the Wnt7b gene in skin tumour cells using CRISPR/Cas9 technology. Inactivation of the Wnt7b gene blocks the soft agar outgrowth of tumour cells to the same extent as addition of C59 and this can be largely rescued by WNT3a conditioned medium (Fig. 6b, c). In line with these observations, shRNA knockdown of Wnt7b gene inactivation is shown by the significantly reduced transcription of the canonical WNT signalling target Axin2 in edited tumour cells which can be rescued by WNT3a supplementation (Supplementary figure 11).

Discussion

Our data indicate that the contribution of WNT signalling to cellular transformation is mediated to a significant extent by stimulating CDK6 activity. Our observations fit a scenario in which p15INK4b plays a critical role in the hair follicle growth cycle.

**Fig. 6** Validation of functional involvement of WNT7b in the transformed nature of MEFs and skin tumour cell lines. a Anchorage-independent colony outgrowth of Cdkn2a KO and Cdkn2ab KO MEFs with and without WNT3a supplementation. b Anchorage-independent colony formation of tumour cell lines 84-5 under regular (−) conditions, in the presence of WNT3a supplementation, in the presence of the porcupine inhibitor C59, in the presence of C59 and WNT3a supplementation. c Anchorage-independent colony formation of tumour cell line 94-1-4 under regular (−) conditions, in the presence of C59 and WNT3a supplementation, in the presence of the porcupine inhibitor C59, in the presence of C59 and WNT3a supplementation. d Anchorage-independent colony formation of tumour cell lines 84-5 and 84-5-W6 knockout for Wnt7b with and without Wnt3a supplementation. Statistical analysis (Unpaired t-test): ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent standard error of the mean (3 biological replicates).
by keeping CDK6 activation through WNT signalling in check. In the absence of p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} this then creates a favourable setting for tumourigenesis.

Unfortunately, graft experiments of the early passage tumour-derived cell lines in immunodeficient NSG mice did not result in tumour outgrowth as is often observed with primary tumours making it impossible to confirm the effects of Wnt7b in this complementary in vivo setting. However, our data are fully in line with a number of published observations. Upregulation of Wnt7b has been reported in hfSCs during physiological and precocious anagen after BMP inhibition\textsuperscript{12}. Furthermore, Wnt7b conditional gene targeting during HF morphogenesis disrupts HF cycling causing a shorter\textsuperscript{12} anagen, premature catagen onset with overall shorter hair. These ‘in vivo’ observations strongly support the stimulating effect of natural Wnt7b overexpression on hfSCs and thereby its oncogenic role in the development TBCs. In addition, clinical data also implicate a contribution of subtle modulation of Wnt7b expression to the development of oral and oesophageal squamous carcinoma\textsuperscript{26,27}.

It is important to emphasize that p15\textsuperscript{Ink4b} is constitutively expressed in the hair follicle, kidney and lung, this in contrast to p16\textsuperscript{Ink4a}. Especially in conditions of high levels of WNT ligands such as is the case for Wnt7b in the hair follicle, CDK6 activation in the absence of p15\textsuperscript{Ink4b} could lead to increased anchorage-independence, migration and proliferation of progenitor cells. The absence of a backup of p16\textsuperscript{Ink4a} or p19\textsuperscript{Arf} to stop cells that respond to these oncogenic signals would increase the risk of accumulating genomic aberrations leading to tumour development.

Loss of Cdkn2b could pose a similar risk in other physiological conditions with allelic variation in WNT signalling and/or CDK6 activation itself. This might apply to kidney development which involves several WNT family members\textsuperscript{28} with concurrent high expression of p15\textsuperscript{Ink4b} (ref.\textsuperscript{17}). Of note, renal cell carcinomas frequently show inactivating mutations in CDKN2b (ref.\textsuperscript{29}) concomitant with WNT pathway activation\textsuperscript{30}. Intriguingly, allelic variants of DKK3, which is implicated in WNT signalling control, have been associated with predisposition to renal cell carcinoma\textsuperscript{31} and squamous lung carcinoma\textsuperscript{32} as well. Furthermore, both p15\textsuperscript{Ink4b} and CDK6 are critically implicated in the regulation of hematopoietic stem cell fate in mouse and man\textsuperscript{33–35}. Interestingly, loss of CDKN2b expression\textsuperscript{36} and enhanced transcription of natural CDK6 alleles are very frequently observed in several leukaemias such as AML and ALL (ref.\textsuperscript{34}). Therefore, CDKN2b deficiency and variation in CDK6 activation could put progenitor cells at risk to become pre-malignant.

Our studies also point to the significance of the deletion of the complete CDKN2ab locus in specific tumour subsets, such as mesothelioma, squamous cell carcinoma, and glioblastoma making it worthwhile to further explore whether WNT and/or CDK6 plays a critical role in these tumours even in the absence of any mutations in these genes. This could motivate a much broader therapeutic application of specific CDK6 and WNT pathway inhibitors.

In conclusion, we here have described a cancer predisposing QTL that poses a very high risk when co-occurring with the frequently observed somatic deletion of the Cdkn2ab tumour suppressor locus. Put in a broader perspective, this might well be a recurrent theme in tumour development with a critical role for WNT signalling to maintain stem cells involved in tissue renewal and regeneration. Their proliferation has been recently coined as anchorage deficiency and variation in CDK6 activation that synergises with Cdkn2ab loss and is required for tumour formation in these mice.
Methods
Animal experiments. All animal experiments comply with international regulations and guidelines and have been authorized by the experimental animal committee at The Netherlands Cancer Institute.

Cdkn2ab mutant alleles. The construction of the Inktab allele (knockout for p15Ink4b and p16Ink4a) and p15Ink4b allele (knockout for p15Ink4b, p19Arf, and p16Ink4a) has been described previously. The Cdkn2ab:Ark mutant allele (knockout for p15Ink4b and conditional knockout for p14Arf) was generated by replacing the Cdkn2ab exon 1 and 2 by a hygromycin expression cassette carrying a 3LoxP site followed by the insertion of a 1LoxP site 3’ of exon 1pC of Cdkn2a. These modifications were performed in 129P2 ES cells using conventional targeting procedures. Mice carrying knockout alleles for Cdkn2ab, Cdkn2a and Cdkn2Abb were obtained by crossing Act-cre mice expressing Cre in the germline (ref. 5) to Inktab, Cdkn2a and Cdkn2Abb, respectively. Additional information on the alleles can be found on the MGI website, accession number 5767459.

Histological analysis. Tissues were fixed in either 4% formalin or acidified formaldehyde (ethanol/acetic acid/formaldehyde/saline at 40:5:10:45 v/v). Sections (4 μm thickness) were deparaffinized and stained with hematoxylin and eosin (H&E) according to standard procedures. For immunohistochemistry (IHC), 4-μm-thick sections were made. Antibodies used in this study are listed in Supplementary Table 4.

Antibody staining was revealed using either diaminobenzidine or 3-amino-9-ethylcarbazole chromogen. Images were captured using a Zeiss Axioskop2 Plus microscope (Carl Zeiss Microscopy) equipped with a Zeiss AxioCam HRc digital camera, and processed using AxioVision 4 software (Carl Zeiss Vision).

DNA profiling. DNA profiling of the back cross mice was performed by a genome-wide scan (autosomal) using a panel of 134 polymorphic microsatellite markers distinguishing allele length between 129P2 and FVB genotypes at a mean density of ~20 Mb (~10 cM). For distinguishing allele length between 129P2 and FVB genotypes at a mean density of ~8 NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-09321-8 | www.nature.com/naturecommunications

β-Catenin dependent transcription assay. Activation of the WNT/β-catenin pathway in Cdkn2a−/− and Cdkn2ab−/− mice was measured using a TOP-FOP assay. One day prior to transient transfection, Cdkn2a−/− and Cdkn2ab−/− mice were seeded in a density of 5.0×104 cell per well of 12-well plate. The TOP-WRE (Wnt-responsive element) reporter plasmid was co-transfected with TOPFLASH (controls) and TOPFLASH/Wnt1 (positive control) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The TOP-FOP assay was performed according to the manufacturer’s instructions.

Protein expression analysis. Cell pellets were lysed in RIPA buffer and the lysates were cleared by centrifugation at 20,000g for 10 min. Proteins were separated on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (Schleicher and Schuell) or Immobilon (Millipore) membranes and probed with antibodies. Immunocomplexes were visualized by enhanced chemiluminescence (GE Healthcare) using goat anti-rabbit (AMO03D, Biosource) or goat anti-mouse (AMO3404, Biosource) IgG secondary antibodies coupled to horseradish peroxidase. The primary antibodies used were: WNT7b: Abcam (ab94915); CDK6: Thermo (AHZ0232).

RNA expression analysis. RNA was isolated from frozen tumour pieces and cell pellets using RNAeasy columns (Qiagen). cDNA was prepared using the Tetro ONE step RT-PCR kit purchased from Bioline (BIO92020) with the following primer sets: Wnt7b: exon 3f 5’-TCACACCTTTCGTGGTCTT-3’; exon 4f 5’-AGGTTCTGTAGTTGTGCTTA-3’. PCR products were sequenced using the ABI Prism 7000 detection system.

Analysis of allele-specific expression. RNA isolation and DNA preparation was performed as described above. Wnt7b mRNAs from 129P2 and FVB/N mice in the 3’ UTR at position 505 in exon 4 (G in 129P2 and C in FVB/N, creating a N1, 490 in the TOP-Flash vector). The TOP-FOP assay was performed according to the manufacturer’s instructions.

Analysis of allele-specific expression. RNA isolation and DNA preparation was performed as described above. Wnt7b mRNAs from 129P2 and FVB/N mice in the 3’ UTR at position 505 in exon 4 (G in 129P2 and C in FVB/N, creating a N1, 490 in the TOP-Flash vector). The TOP-FOP assay was performed according to the manufacturer’s instructions.

β-Catenin dependent transcription assay. Activation of the WNT/β-catenin pathway in Cdkn2a−/− and Cdkn2ab−/− mice was measured using a TOP-FOP assay. One day prior to transient transfection, Cdkn2a−/− and Cdkn2ab−/− mice were seeded in a density of 5.0×104 cell per well of 12-well plate. The TOP-WRE (Wnt-responsive element) reporter plasmid was co-transfected with TOPFLASH (controls) and TOPFLASH/Wnt1 (positive control) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The TOP-FOP assay was performed according to the manufacturer’s instructions.

Protein expression analysis. Cell pellets were lysed in RIPA buffer and the lysates were cleared by centrifugation at 20,000g for 10 min. Proteins were separated on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes (Schleicher and Schuell) or Immobilon (Millipore) membranes and probed with antibodies. Immunocomplexes were visualized by enhanced chemiluminescence (GE Healthcare) using goat anti-rabbit (AMO03D, Biosource) or goat anti-mouse (AMO3404, Biosource) IgG secondary antibodies coupled to horseradish peroxidase. The primary antibodies used were: WNT7b: Abcam (ab94915); CDK6: Thermo (AHZ0232).

RNA expression analysis. RNA was isolated from frozen tumour pieces and cell pellets using RNAeasy columns (Qiagen). cDNA was prepared using the Tetro ONE step RT-PCR kit purchased from Bioline (BIO92020) with the following primer sets: Wnt7b: exon 3f 5’-TCACACCTTTCGTGGTCTT-3’; exon 4f 5’-AGGTTCTGTAGTTGTGCTTA-3’. PCR products were sequenced using the ABI Prism 7000 detection system.

Analysis of allele-specific expression. RNA isolation and DNA preparation was performed as described above. Wnt7b mRNAs from 129P2 and FVB/N mice in the 3’ UTR at position 505 in exon 4 (G in 129P2 and C in FVB/N, creating a N1, 490 in the TOP-Flash vector). The TOP-FOP assay was performed according to the manufacturer’s instructions.

β-Catenin dependent transcription assay. Activation of the WNT/β-catenin pathway in Cdkn2a−/− and Cdkn2ab−/− mice was measured using a TOP-FOP assay. One day prior to transient transfection, Cdkn2a−/− and Cdkn2ab−/− mice were seeded in a density of 5.0×104 cell per well of 12-well plate. The TOP-WRE (Wnt-responsive element) reporter plasmid was co-transfected with TOPFLASH (controls) and TOPFLASH/Wnt1 (positive control) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The TOP-FOP assay was performed according to the manufacturer’s instructions.
172 of exon 15 (rev: TGCCTTGGGATGGCTG). PCR products were sequenced and scored for C or A at position 172 of exon 15.

**CRISPR/Cas Gene editing.** Tumour cell lines were modified according to standard procedures involving transfections of pX330 vectors expressing Cas9 and specific guideRNAs (gRNA) (ref. 39). Transfections were done using Lipofectamine LTX from Invitrogen (15338–100). gRNA designs were obtained from the CRISPR Design website from MIT (http://crispr.mit.edu). After transient selection for puromycin resistance clones were picked, expanded and molecularly characterized. The Wnt7b gene was inactivated by co-transfection of two pX330 vectors expressing gRNAs enabling the excision of exon 3. Sequence gRNA1: AGGTTCAATCAGTGCTGA, sequence gRNA2: CGGGCGAGAAAGTTGACGGTT. Editing was confirmed by PCR analysis using primers around the deleted exon 3. The Pdgfrb gene was inactivated by co-transfection of two pX330 expressing gRNAs enabling the excision exon 2. Editing was confirmed by PCR analysis using primers around the deleted exon 2. gRNA1: TCTCCTGTGTCAGCCAGACAG, sequence gRNA2: AGGGAGCTGCCAGATTACCTA. The Cdk6 gene was inactivated by co-transfection of two pX330 expressing gRNA enabling the excision of part of exon 3. Sequence gRNA1: GCCCGCGACCTGAAGAACGG, sequence gRNA2: GACCTTGAGGACCCAGCG. Editing was confirmed by PCR analysis using primers around the deleted part of exon 3.

**ShRNA knockdown.** Lentiviral constructs (pLKO.1 vector) expressing shRNAs against Wnt7b and Cdk6 were obtained from The RNAi Consortium (Broad Institute/Harvard). Plasmid preparation, virus production and virus infection were performed according published protocols. Sequence of shRNA: Wnt7b-4: GCT ACATTCTTGCCGGCTAT; sequence of shRNA Wnt7b-7: CGCGGCAATTGT ATCCGGTG; sequence of shRNA Cdk6-1: CCGGTTGACATTTTGCAAGAA; sequence of shRNA Cdk6-2: ATCTTCTGAGAAAACCTACTT. Supplementary Table 3 provides the sequences of primers used in the experiments described in the Methods section above.

**Data availability.** The exome sequencing data of skin tumours and its matched controls have been deposited in the NCBI Sequence Read Archive with accession code PRJEB8102. Chipseq data are available from the Gene Expression Omnibus under accession code GSE125885. The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary information files or available from the authors upon request.

Received: 18 May 2018 Accepted: 13 February 2019
Published online: 29 March 2019

**References**

1. Gil, J. & Peters, G. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat. Rev. Mol. Cell. Biol. 7, 667–677 (2006).
2. Krümpenfort, P. et al. p51InkB is a critical tumour suppressor in the absence of p16Ink4a. Nature 448, 943–946 (2007).
3. Carter, H. et al. Interaction Landscape of Inherited Polymorphisms with specific guideRNAs (gRNA) (ref. 39). Transfections were done using Lipofectamine LTX from Invitrogen (15338–100). gRNA designs were obtained from the CRISPR Design website from MIT (http://crispr.mit.edu). After transient selection for puromycin resistance clones were picked, expanded and molecularly characterized. The Wnt7b gene was inactivated by co-transfection of two pX330 vectors expressing gRNAs enabling the excision of exon 3. Sequence gRNA1: AGGTTCAATCAGTGCTGA, sequence gRNA2: CGGGCGAGAAAGTTGACGGTT. Editing was confirmed by PCR analysis using primers around the deleted exon 3. The Pdgfrb gene was inactivated by co-transfection of two pX330 expressing gRNAs enabling the excision exon 2. Editing was confirmed by PCR analysis using primers around the deleted exon 2. gRNA1: TCTCCTGTGTCAGCCAGACAG, sequence gRNA2: AGGGAGCTGCCAGATTACCTA. The Cdk6 gene was inactivated by co-transfection of two pX330 expressing gRNA enabling the excision of part of exon 3. Sequence gRNA1: GCCCGCGACCTGAAGAACGG, sequence gRNA2: GACCTTGAGGACCCAGCG. Editing was confirmed by PCR analysis using primers around the deleted part of exon 3.

**ShRNA knockdown.** Lentiviral constructs (pLKO.1 vector) expressing shRNAs against Wnt7b and Cdk6 were obtained from The RNAi Consortium (Broad Institute/Harvard). Plasmid preparation, virus production and virus infection were performed according published protocols. Sequence of shRNA: Wnt7b-4: GCT ACATTCTTGCCGGCTAT; sequence of shRNA Wnt7b-7: CGCGGCAATTGT ATCCGGTG; sequence of shRNA Cdk6-1: CCGGTTGACATTTTGCAAGAA; sequence of shRNA Cdk6-2: ATCTTCTGAGAAAACCTACTT. Supplementary Table 3 provides the sequences of primers used in the experiments described in the Methods section above.

**Data availability.** The exome sequencing data of skin tumours and its matched controls have been deposited in the NCBI Sequence Read Archive with accession code PRJEB8102. Chipseq data are available from the Gene Expression Omnibus under accession code GSE125885. The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary information files or available from the authors upon request.
Transgenic Core Facility, Oscar Krijgsman and Koen Schipper, The Netherlands Cancer Institute, for advice. We thank Jan van der Vliet, Miranda Cozijnse and members of the animal facility of the Netherlands Cancer Institute for maintaining the mice. We thank members of the Department of Experimental Animal Pathology and members of the Sequencing Facility of the Netherlands Cancer Institute. This work was supported the Queen Wilhelmina Prize from the Dutch Cancer Society to A.B, by the European Research Council under the European Union’s Seventh Framework Programme (FP7/ 2007-2013)/ERC synergy grant agreement n° 319661 COMBATCANCER of which A.B. is one of the principal investigators, and a National Roadmap grant for Large-Scale Research Facilities of the Netherlands Organization for Scientific Research.

**Author contributions**

M.S. performed and analysed microsatellite mapping; J-P.L. was involved in the tissue culture and biochemical experiments; J-Y.S. was responsible for the histopathological analysis; R.B. and D.J.A. were involved in the exome sequencing and CNV analysis; R.v. W., H.T. and E.d.W. performed and analysed the Chipseq experiments; P.K. was responsible for the design and execution of the experiments; P.K. and A.B. wrote the paper.

**Additional information**

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-09321-8.

**Competing interests:** The authors declare no competing interests.

**Reprints and permission** information is available online at http://npg.nature.com/reprintsandpermissions/

**Journal peer review information:** Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.