Antagonistic TSC22D1 variants control BRAF^{E600}-induced senescence

Cornelia Hömig-Hölzel¹, Remco van Doorn^{1,4}, Celia Vogel¹, Markus Germann², Marco G Cecchini², Els Verdegaal³ and Daniel S Peeper^{1,*}

¹Division of Molecular Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands, ²Department of Urology and Clinical Research, Urology Research Laboratory, University of Bern, Bern, Switzerland and ³Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands

Oncogene-induced cellular senescence (OIS) is an increasingly recognized tumour suppressor mechanism that confines the outgrowth of neoplastic cells *in vivo*. It relies on a complex signalling network, but only few components have been identified so far. Gene-expression profiling revealed a >100-fold increase in the levels of the transcription factor and putative tumour suppressor gene TGF β -stimulated clone 22 (*TSC22D1*) in BRAF^{E600}-induced senescence, in both human fibroblasts and melanocytes. Only the short *TSC22D1* transcript was upregulated, whereas the abundance of the large protein variant was suppressed by proteasomal degradation. The *TSC22D1* protein variants, in complex with their dimerization partner TSC22 homologue gene 1 (THG1), exerted opposing functions, as selective depletion of the short form, or conversely, overexpression of the large variant, resulted in abrogation of OIS. This was accompanied by the suppression of several inflammatory factors and p15^{INK4B}, with *TSC22D1* acting as a critical effector of C/EBP β . Our results demonstrate that the differential regulation of antagonistic *TSC22D1* variants is required for the establishment of OIS and suggest distinct contributions of *TSC22* family members to the progression of BRAF^{E600}-driven neoplasia.

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Introduction

Tissue homeostasis of multicellular organisms requires a well-controlled balance of cell death and renewal. This

capacity is essential for survival, but also favours the accumulation of mutations that may ultimately lead to the outgrowth of malignant cell populations. Given the enormous amount of cell renewal throughout lifetime, powerful failsafe mechanisms must exist to restrain the expansion of cells with acquired oncogenic mutations to keep the incidence of cancer at low rates. Apoptosis and lack of vessel formation help to prevent malignant transformation and expansion of cancerous cells (Lowe *et al*, 2004; Sherr, 2004). Oncogene-induced cellular senescence (OIS) is now considered to be another major barrier against tumourigenesis (Campisi, 2005; Adams, 2009). Activated oncogenes like RAS and BRAF in non-transformed cells trigger a proliferation arrest response that resembles replicative senescence, requiring a network of tumour suppressors and other factors, many of which remain to be identified. Cellular senescence is characterized by a lack of proliferation, induction of various biomarkers including elevated senescence-associated (SA) β -galactosidase activity and increased expression of tumour suppressor genes, such as p15^{INK4B} and p16^{INK4A} (Collado and Serrano, 2010; Kuilman *et al*, 2010).

An additional hallmark of senescence is the presence of SA heterochromatic foci (SAHF), structures of heterochromatin that contribute to the stable repression of proliferation-associated genes (Narita *et al*, 2003). Consistently, SAHF formation is reduced in cells that bypass OIS, for example by loss of p53 or C/EBP β (Ye *et al*, 2007; Zhang *et al*, 2007; Kuilman *et al*, 2008). In agreement with a role for heterochromatin in senescence, *N-Ras*^{V12}-transgenic mice deficient for the histone methyltransferase *Suv39h1* die from invasive T-cell lymphomas (Braig *et al*, 2005). HIRA (a histone chaperone) translocation to PML (promyelocytic leukaemia) nuclear bodies also contributes to senescence (Zhang *et al*, 2005; Adams, 2007, 2009). These subnuclear structures enable the formation of ASF1a-containing complexes and contribute to SAHF formation (Zhang *et al*, 2005; Narita *et al*, 2006; Ye *et al*, 2007). The DNA damage response (DDR) has been suggested to contribute to senescence as well (Bartkova *et al*, 2006; Di Micco *et al*, 2006; Mallette *et al*, 2007). Other processes, including ER stress, reactive oxygen species production and autophagy have also been linked to senescence (Denoyelle *et al*, 2006; Lu and Finkel, 2008; Moiseeva *et al*, 2009; Young *et al*, 2009). Senescence can be triggered not only by oncogene activation, but also by the loss of tumour suppressors, as has been shown, for example, for PTEN and NF-1 (Chen *et al*, 2005; Courtois-Cox *et al*, 2006).

Even though OIS was first described *in vitro* (Serrano *et al*, 1997), over the last 5 years several studies have demonstrated that OIS functions *in vivo* to suppress tumourigenesis (Braig *et al*, 2005; Chen *et al*, 2005; Collado *et al*, 2005; Michaloglou *et al*, 2005; Bartkova *et al*, 2006; Courtois-Cox *et al*, 2006; Gray-Schopfer *et al*, 2006; Dankort *et al*, 2007). For example, nevi (moles), common benign tumours of melanocytes that frequently harbour activating mutations in BRAF (most commonly BRAF^{E600}), display characteristics of

*Corresponding author. Division of Molecular Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, Room P1.011, Amsterdam 1066 CX, The Netherlands. Tel.: +31 20 5122 002; Fax: +31 20 5122 011; E-mail: d.peeper@nki.nl

⁴Present address: Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands

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senescence, in both humans and BRAF^{E600} knock-in mice (Michaloglou *et al*, 2005; Gray-Schopfer *et al*, 2006; Dankort *et al*, 2007; Dhomen *et al*, 2009). Therefore, OIS acts in human nevi to permanently arrest melanocytes suffering from an oncogenic mutation, preventing melanomagenesis. Although the melanoma-susceptibility gene *CDKN2A* (encoding p16^{INK4A}) is commonly induced by BRAF^{E600}, immunohistochemical and genetic evidence in mice and humans, as well as cultured cells, indicates that the senescence response does not critically depend on it (Michaloglou *et al*, 2005; Dhomen *et al*, 2009; Haferkamp *et al*, 2009; Kuilman *et al*, 2010). This suggests that other, yet to be identified, genes with tumour suppressor functions contribute to the establishment of OIS. Indeed, using an unbiased gene-expression profile analysis, we have previously identified a crucial role for the inflammatory transcriptome, including cytokines like IL6 and IL8 (Kuilman *et al*, 2008). The transcription factor C/EBP β has been shown to coordinate the upregulation of IL6 and IL8 in response to BRAF^{E600}, as well as HRAS^{V12}, and is critically required for OIS (Sebastian *et al*, 2005; Kuilman *et al*, 2008; Atwood and Sealy, 2009). Loss of IGFBP7 or CXCR2 also results in bypass of BRAF^{E600}-induced senescence (Acosta *et al*, 2008; Wajapeyee *et al*, 2008). The CXCR2 receptor transmits signals from various CXC chemokine family members like IL8 and GRO1 (CXCL1/GRO α). IGFBP7 belongs to a group of proteins that bind and neutralize members of the insulin-like growth factors (IGFs). Thus, secreted factors are important mediators of both oncogene-induced and replicative senescence, collectively termed as the senescence-messaging secretome (SMS) (Kuilman and Peeper, 2009). Some of those depend on a persistent DDR, and this phenomenon has been termed senescence-associated secretory phenotype (SASP) (Coppe *et al*, 2008; Rodier *et al*, 2009).

These examples illustrate that the establishment of OIS requires a complex signalling network that we have only begun to dissect. Apparently, the removal of critical nodes can cause the senescence program to collapse, thereby paving the way for oncogenic transformation. In view of the key role of OIS in tumour suppression, it is imperative that such factors are discovered. Here, we have used gene-expression profiling to screen for novel critical mediators of OIS, and describe the identification of a carefully regulated transcription factor, TSC22D1, which serves as a critical component of the C/EBP β pathway regulating OIS.

Results

TSC22D1.2 is expressed as a function of OIS

To identify new mediators of OIS, we have previously developed a screening system that allows for the identification of genes whose transcription is induced in cells undergoing senescence upon exposure to oncogenic BRAF^{E600}, and whose levels decline when OIS is abrogated (Figure 1A). This has led to the identification of the inflammatory transcriptome as a novel and critical mediator of OIS (Kuilman *et al*, 2008). For this previous study, we had stratified the gene-expression profiles by gene ontology (GO) annotation. Although powerful to uncover large functional families, this approach may preclude the identification of potentially important and significantly regulated single genes that fall into GO classes that are not significantly deregulated. Looking

specifically at single genes, which are strongly induced by oncogenic stress in comparison to proliferating and quiescent control cells, and which are significantly reduced in cells whose OIS program is abrogated, we found the *TSC22D1* gene to fulfill these criteria. It also attracted our attention, because it was previously shown to be a RAS-responsive gene (Mason *et al*, 2004). Furthermore, TSC22D1 was originally described as TGF β -responsive transcription factor, comprising a TSC box (a highly conserved family specific domain) and a leucine zipper domain, which enables the protein to form homodimers or heterodimers with the product of the closely related product encoded by the TSC22 homologue gene 1 (THG1; also called TSC22D4) (Shibanuma *et al*, 1992; Ohta *et al*, 1996; Kester *et al*, 1999). Finally, based on its elevated expression in premalignant lesions and loss at advanced cancer stages, *TSC22D1* has previously been speculated to be a tumour suppressor gene (Nakashiro *et al*, 1998; Iida *et al*, 2005; Shostak *et al*, 2005; Rentsch *et al*, 2006). It exerts a pro-apoptotic function in gastric cancer cell lines (Ohta *et al*, 1997; Uchida *et al*, 2000) and its disruption in mice causes increased proliferation and faster repopulation of haematopoietic precursors (Yu *et al*, 2009). However, a functional role of TSC22D1 in OIS has not been reported.

Two protein-coding transcripts of *TSC22D1* have been described (Shibanuma *et al*, 1992; Fiol *et al*, 2007), *TSC22D1.1* and *TSC22D1.2* (this will be discussed in more detail below). In the microarray analysis, we found specifically the *TSC22D1.2* transcript to be induced. The *TSC22D1.2* mRNA was upregulated >100-fold in senescent BRAF^{E600}-expressing human diploid fibroblasts (HDFs), as judged by quantitative real-time RT-PCR (Figure 1B). Further confirming our microarray results, *TSC22D1.2* levels were hardly elevated when OIS was abrogated (owing to co-expression of CDC42^{V12}; Kuilman *et al*, 2008). It was not regulated in cells rendered quiescent by serum depletion, indicating that cell-cycle arrest *per se* is insufficient to induce *TSC22D1.2*. This observation suggests a specific link between *TSC22D1.2* and OIS. Also *TSC22D1.2* proteins were highly induced during OIS, whereas they were hardly detectable in control cells (Figure 1C). The nature of the various *TSC22D1.2* gene products will be discussed below. *TSC22D1.2* mRNA and protein levels accumulated also strongly in BRAF^{E600}-expressing human melanocytes, indicating that this regulation is not a cell type-dependent phenomenon (Figure 1D and E).

Differential start codon usage of the TSC22D1.2 transcript

Most studies on *TSC22D1.2* have been focusing on the longest encoded protein of 144 amino acids (aa) with a predicted size of 18 kDa (Figure 2A, red triangle). However, by western blotting with a TSC22D1-specific monoclonal antibody, we detected two additional major proteins migrating with apparent molecular weights of ~17 and 14 kDa, in both senescent HDFs and melanocytes (Figures 1C and E and 2A). These forms could be either proteolytic degradation products or protein variants of TSC22D1. The *TSC22D1.2* transcript harbours two additional potential in-frame start codons (Figure 2C). The smallest transcript has previously been described and produces an 86-aa protein (Khoury *et al*, 2008; Figure 2A, black triangle). Furthermore, a 134-aa product would be encoded by a transcript using the middle start codon (Figure 2A, green triangle).

Figure 1 TSC22D1.2 is expressed as a function of OIS. (A–C) Cycling HDF (Tig3(et)) were transduced with control or CDC42^{V12}-expressing retrovirus. After brief pharmacological selection they were transduced with a BRAF^{E600}-expressing retrovirus. Cells undergoing OIS (upon transduction with BRAF^{E600}), bypassing OIS (CDC42^{V12} in combination with BRAF^{E600} expression), cycling as well as serum-starved cells (for 48 h) were used for gene-expression microarray analysis. The experiment was performed in independent duplicates (series 1; 2). (A) Cell proliferation assay performed in parallel to the microarray analysis. (B) TSC22D1.2 regulation in HDF, as determined by qRT-PCR. The two series represent the two independent experiments that were used for the microarray analysis. Levels of transcripts are normalized to those found in control cells in the first series. (C) Immunoblot for the detection of TSC22D1.2 in HDF carrying the indicated constructs, 8 days after BRAF^{E600} expression. β-Actin serves as a loading control. (D, E) Cycling human melanocytes (FM 186) were transduced using lentivirus encoding BRAF^{E600} or control virus. Four days after transduction, cells were harvested and TSC22D1.2 transcript and protein levels were assessed by qRT-PCR and immunoblotting. β-Actin serves as a loading control. (D) TSC22D1.2 regulation in melanocytes, as determined by qRT-PCR, based on three independent experiments; error bars represent s.d. Transcript levels were normalized to those found in control cells in the first series. (E) TSC22D1.2 protein levels were analysed by immunoblotting using a TSC22D1-specific antibody. Arrowheads indicate TSC22D1.2 proteins of 144 aa (18 kDa), 134 aa (17 kDa) (the double band of 144 and 134 aa can be better seen in Figure 2A) and 86 aa (14 kDa). β-Actin serves as a loading control.

To clarify the origins of the three TSC22D1.2 polypeptides seen in senescent cells, we generated several cDNA-expression vectors to drive expression from the different translational start sites. The TSC22D1.2a vector variant (labelled red) contains an artificial optimal Kozak sequence preceding the

first ATG (ATG¹⁴⁴) to stimulate translational initiation from this start site. Using this construct, we observed only a single species of TSC22D1.2, which exactly co-migrated with the weakly expressed endogenous 18 kDa product seen in OIS (Figure 2B). In the TSC22D1.2b construct, we used the

Figure 2 Differential start codon usage of the *TSC22D1.2* transcript. (A) Immunoblot for TSC22D1.2 of HDF with or without BRAF^{E600}, 8 days after BRAF^{E600} expression. β-Actin serves as a loading control. (B) To express TSC22D1.2, three different constructs were created. TSC22D1.2a has an optimal Kozak sequence in front of the first ATG to initiate translation of a 144-aa protein; TSC22D1.2b contains the nine base pairs of the genomic locus in front of the first ATG, enabling the start of translation similar to the wild-type situation; in TSC22D1.2c the second ATG is mutated to TTG, leading to the expression mainly of an 86-aa protein. All three variants can be depleted by specific shRNAs, confirming the identity of the proteins (see Supplementary Figure S8). Immunoblot for TSC22D1.2 of HDF transduced with the various TSC22D1.2 constructs. CDK4 serves as a loading control. (C) Schematic drawing of the TSC22D1.2 protein. The TSC box, a highly conserved domain found in all TSC22 protein family members, is depicted in blue. The grey box indicates the leucine zipper domain. Arrowheads indicate the translational start sites of the 144-aa (red), 134-aa (green) and 86-aa (black) polypeptides along the *TSC22D1.2* transcript.

endogenous nine bases in front of the first ATG as a Kozak sequence. Cells expressing the TSC22D1.2b construct (labelled green) produced predominantly the two smaller forms of TSC22D1.2, migrating at 17 and 14 kDa, which precisely matched the major protein variants found in BRAF^{E600}-expressing cells (Figure 2B). Finally, we used the TSC22D1.2b vector as a template and converted the second ATG (ATG¹³⁴) into TTG (encoding leucine). This construct, encoding TSC22D1.2c (labelled black), produced predominantly a 14 kDa protein that co-migrated with the smallest TSC22D1.2 product seen in OIS. Proteasome inhibition increased, rather than decreased the levels of the smallest TSC22D1.2 proteins running at 14 and 17 kDa, suggesting that they did not correspond to degradation products of larger TSC22D1 species (data not shown). These findings strongly suggest that the two additional start codons within the TSC22D1.2 transcript are naturally used during OIS and result in the production of proteins of 134 and 86 aa.

Despite the strong induction of TSC22D1.2 seen in senescent cells, its overexpression failed to inhibit proliferation of HDFs, independent of their p16^{INK4A} status (Supplementary Figure S1A). Consistent with this, the protein levels of several cell-cycle regulators and transcripts of senescence-regulated genes like IL6, IL8 and IL1β were also not altered in a major way (Supplementary Figure S1B and C).

TSC22D1 is crucial for the induction of OIS

To examine whether TSC22D1 is functionally involved in OIS, we depleted it using two non-overlapping short-hairpin (sh) RNAs (sh-TSC22(1) and sh-TSC22(2)). We confirmed the strong suppression of the TSC22D1.2 mRNA and protein by

the respective shRNAs by qRT-PCR and immunoblotting (Figure 3A and B). Even though p16^{INK4A} is not strictly required for BRAF^{E600}-induced senescence in HDFs, it may contribute to it in the context of additional factors (Michaloglou *et al*, 2005; Kuilman *et al*, 2008). Therefore, we generated a polyclonal HDF (Tig3) fibroblast cell line expressing an shRNA targeting p16^{INK4A} (Tig3(et)-16i), which was used for most follow-up studies with HDF. Importantly, knockdown of TSC22D1 enabled the cells to abrogate BRAF^{E600}-induced senescence and to continue active proliferation, as shown by long-term proliferation and BrdU incorporation assays (Figure 3C (upper panel) and D). This was independent of the p16^{INK4A} status as it was seen irrespective of p16^{INK4A} depletion (Supplementary Figure S2A). Furthermore, the BRAF^{E600} expression and activation of its downstream effectors was equal in all samples (Supplementary Figure S3). OIS abrogation by TSC22D1 depletion was associated with a marked reduction in SA β-galactosidase activity (Figure 3C, middle panel). TSC22D1-knockdown cells also failed to show the typical BRAF^{E600} senescence-associated spindle-shaped morphology (Figure 3C, lower panel). Consistently, the degree of depletion of TSC22D1 transcript and protein by the individual shRNAs correlated well with the extent of the observed bypass phenotype, with shRNA(2) giving the most profound effects.

Of many cell-cycle proteins analysed, we found that only the levels of p15^{INK4B} were downregulated by TSC22D1 depletion, consistent with our previous results on the C/EBPβ-interleukin pathway (Supplementary Figure S3; Kuilman *et al*, 2008). The observation that only a proportion of cells bypassing senescence stain negatively for SA β-galactosidase is most likely due to the fact that the levels of

Figure 3 TSC22D1 is crucial for the induction of OIS. **(A)** HDF (Tig3(et)-16i) expressing two independent hairpins against TSC22D1 (sh-TSC22(1)/(2)) were exposed to BRAF^{E600} for 8 days. Amount of *TSC22D1.2* transcript was analysed by qRT-PCR. Transcript levels from three independent experiments were normalized to those found in non-senescent control cells and are represented as mean with s.d. **(B)** The samples described in **(A)** were analysed by immunoblotting for the amount of TSC22D1 protein. CDK4 serves as loading control, arrowheads indicate the three TSC22D1.2 proteins (see Figures 1C and 2A). **(C)** Cell proliferation assay of HDF described in **(A)**. Cells were fixed and stained 10 days after exposure to BRAF^{E600} (upper row). Representative images of senescence-associated (SA) β -galactosidase staining (middle row) and phase contrast (lower row) are shown for the cells described in **(A)**. Quantification was performed for three independent experiments, with s.d. **(D)** Nine days after exposure to BRAF^{E600} BrdU incorporation was measured in the samples described in **(A)**, after a 3-h BrdU pulse (measured by FACS). Levels are represented as mean from at least three independent experiments. Error bars represent s.d. **(E)** Cell proliferation assay of FM186 human melanocytes. Cells were lentivirally transduced with the indicated constructs, fixed and stained 8 days after BRAF^{E600} expression. The lower row represents phase contrast images of the cells described in **(E)**.

TSC22D1 were not sufficiently reduced in all cells, probably by differences in viral integration rates or stochastic effects within the cell population. Supporting this explanation, p15^{INK4B} protein levels in cells bypassing senescence were decreased, but never fully reduced to the levels seen in proliferating control cells. Suppression of *TSC22D1* allowed also another HDF strain (IMR90) as well as human melanocytes (FM186) to override BRAF^{E600}-induced senescence (Figure 3E; Supplementary Figure S2B; note that in melanocytes in the absence of BRAF^{E600}, TSC22D1 depletion slowed down proliferation; in spite of this, TSC22D1 depletion in the context of BRAF^{E600} allowed for OIS abrogation). Thus, TSC22D1 appears to have an essential function in BRAF^{E600}-induced senescence, across cell strains and types and independently of p16^{INK4A}.

TSC22D1 acts downstream of C/EBP β on the inflammatory secretome

Next, we asked whether TSC22D1.2 interconnects with the established signalling networks underlying BRAF^{E600}-induced senescence, particularly the one supporting the induction of the SMS. We and others have previously demonstrated that the transcription factor C/EBP β has a key role downstream of the BRAF^{E600}-MEK signalling cascade to orchestrate the OIS response, by regulating the expression of several genes such as *IL6* and *IL8* (Acosta *et al*, 2008; Kuilman *et al*, 2008; Atwood and Sealy, 2009; Figure 4A). Therefore, we analysed the role of C/EBP β in the regulation of TSC22D1 levels. Suppression of C/EBP β by RNAi completely prevented the induction of TSC22D1 mRNA and protein by BRAF^{E600} (Figure 4B–D). Conversely, depletion of TSC22D1 had no

Figure 4 TSC22D1 acts downstream of C/EBPβ on the inflammatory secretome. (A) Cell proliferation assay of HDF (Tig3(et)-16i) expressing the indicated constructs. Cells were fixed and stained 10 days after BRAF^{E600} expression. (B, C, E–G) Regulation of *TSC22D1.2*, *C/EBPβ*, *IL8*, *IL6* and *IL1β* transcripts of the samples described in (A) as determined by qRT-PCR. Measurements are based on at least three independent experiments and standardized to the BRAF^{E600}-expressing senescent cells. Error bars indicate s.d. (D) Protein levels of TSC22D1, BRAF and C/EBPβ were analysed by immunoblotting using the samples described in (A) (arrowheads indicate the three TSC22D1.2 proteins (see Figure 1D), open triangles indicate C/EBPβ proteins). β-Actin serves as a loading control. (H, I) Microarray gene-expression data of BRAF^{E600}-expressing senescent cells were correlated to the expression profile found in cells bypassing BRAF^{E600}-induced arrest upon depletion of either TSC22D1 or C/EBPβ. (H) Two-sample correlation plot. The Pearson product-moment coefficient, reflecting the degree of linear relationship between two variables, is 0.56. (I) Venn diagram displaying the amount of significantly regulated genes with a *P*-value < 0.02. In all, 47 702 transcripts were not significantly regulated. No anti-regulated genes were found.

significant influence on the expression levels of C/EBPβ (Figure 4C and D). These observations indicate that TSC22D1.2 is expressed as a function of C/EBPβ, raising the possibility that the latter serves as a direct transcription factor for the first. However, although there are two potential C/EBPβ-binding sites preceding the first exon of *TSC22D1.2*, we were unable to detect significant promoter binding by chromatin immunoprecipitation (ChIP; data not shown). This

argues for an indirect regulation of TSC22D1 by C/EBPβ, but the intermediary factor(s) remains to be found.

Next, we examined whether TSC22D1 affects the expression of known regulators of OIS. Several members encoded by the senescence-associated transcriptome, including IL1β, IL6 and IL8, were significantly reduced in TSC22D1-depleted cells (Figure 4E–G). Given that C/EBPβ controls transcription of *IL6* and *IL8* by direct promoter binding (Kuilman *et al*,

2008), these results raise the possibility that TSC22D1.2 accounts for the regulation of a proportion of the C/EBP β -directed senescence-associated transcriptome. To investigate this, we compared the gene-expression profiles of BRAF^{E600}-expressing senescent cells with those of TSC22D1- or C/EBP β -knockdown cells that have bypassed OIS. Indeed, we found that, remarkably, 79% (140 out of 178) of the TSC22D1-dependent transcripts were also regulated by C/EBP β (Figure 4H and I). This analysis also showed that, conversely, C/EBP β controls a large set of genes independently of TSC22D1. No anti-regulated genes were detected, suggesting that TSC22D1 and C/EBP β act in an overlapping pathway, with TSC22D1 covering a substantial proportion of the C/EBP β -controlled transcriptome.

Selective induction of the TSC22D1.2 transcript is linked to OIS

As mentioned before, two protein-coding transcripts have been described (Shibanuma *et al*, 1992; Fiol *et al*, 2007). We could detect both, *TSC22D1.1* and *TSC22D1.2*, in HDF (Figure 5A and B). *TSC22D1.1* encodes a protein of 1073 aa, whereas the smaller *TSC22D1.2* transcript codes for 144 and 86 aa polypeptides, and as suggested by our data, also a 134-aa protein. Interestingly, whereas the short *TSC22D1.2* transcript was strongly upregulated in OIS, *TSC22D1.1* was not (Figure 5B). In fact, while the TSC22D1.2^{86aa} and TSC22D1.2^{134aa} proteins were strongly induced, the expression levels of TSC22D1.1^{1073aa} protein even declined upon establishment of OIS (Figure 5C). TSC22D1.1^{1073aa} protein levels could be fully restored upon treatment of senescent cells with the proteasome inhibitor MG132 (Figure 5D). This indicates that while the induction of *TSC22D1.2* is regulated through increased transcription, TSC22D1.1^{1073aa} protein levels are negatively controlled by proteasomal degradation.

The gradual loss of the TSC22D1.1^{1073aa} variant suggests that, in contrast to the smaller TSC22D1.2 proteins, it is not required for the maintenance of OIS. However, as it is expressed early upon BRAF^{E600} expression (Figure 5C, day 6), it might have a role in the initiation of OIS. To address this possibility, we dissected the specific contribution of the *TSC22D1* transcripts to the establishment of OIS. The sh-TSC22(1) and sh-TSC22(2) RNAs target all forms of *TSC22D1*. Since only the small transcript (*TSC22D1.2*) was induced by BRAF^{E600}, we generated two additional knockdown vectors, sh-TSC22(3) targeting the *TSC22D1.2* transcript and, conversely, sh-TSC22(4) specifically targeting the *TSC22D1.1* transcript (Figure 5E). This was confirmed by immunoblotting, demonstrating that only the TSC22D1.2 protein variants were depleted by sh-TSC22(3), but not the 1073-aa protein (Figure 5F; note that this could be seen clearly only in the vector-infected cells, as the levels of this protein strongly decrease upon BRAF^{E600} expression), while sh-TSC22(4) directed the specific depletion of TSC22D1.1 but not TSC22D1.2. As predicted, suppression of only the small TSC22D1.2 protein variants was sufficient to abrogate BRAF^{E600}-induced cell-cycle arrest, to an extent that was similar to that seen for shRNAs targeting both *TSC22D1* transcripts (Figure 5G), and independent of p16^{INK4A} status (Supplementary Figure S4). In contrast, cells depleted specifically for TSC22D1.1 were unable to abolish senescence. We conclude, therefore, that the small TSC22D1.2 protein variants, which are strongly induced by BRAF^{E600}, are

also critically required for OIS, whereas the TSC22D1.1^{1073aa} protein is not.

Ectopic expression of TSC22D1^{1073aa} overrides OIS

Given the contrary regulation of expression of the TSC22D1.1 and TSC22D1.2 protein variants during OIS, we hypothesized that they might have opposing functions. In fact, this has been suggested previously by genetic rescue experiments in *Drosophila* (Gludener *et al*, 2008). We showed above that depletion of the small TSC22D1.2 proteins is sufficient to abrogate OIS. Therefore, we asked whether, conversely, constitutive expression of the large TSC22D1.1 protein would interfere with the establishment of OIS. Indeed, ectopic expression of TSC22D1.1 effectively abolished senescence in HDFs with and without p16^{INK4A} (Figure 6A and B; Supplementary Figure S5). As expected, overexpression of the small TSC22D1.2 proteins failed to do this, in line with our finding that depletion of the small TSC22D1 variants overrides OIS instead. Taken together, these results demonstrate that in OIS the large and small protein variants of TSC22D1 are differentially regulated and have antagonistic functions.

TSC22D1 regulation of OIS involves its dimerization partner THG1

TSC22D1 can form homodimers, but also heterodimers with the closely related TSC22 family member THG1 (Kester *et al*, 1999). We found that THG1 is expressed in HDF and melanocytes, but is not regulated in BRAF^{E600}-induced senescence or in cells bypassing senescence by depletion of TSC22D1 or C/EBP β (Figure 7A). To examine whether THG1/TSC22D1 heterodimer formation occurs in OIS, we immunoprecipitated endogenous THG1 from proliferating and senescent fibroblasts, and probed for co-precipitated TSC22D1 by immunoblotting with a pan-TSC22 family antibody. TSC22D1.1^{1073aa}, TSC22D1.2^{144aa} and TSC22D1.2^{134aa} all efficiently co-precipitated with THG1 (Figure 7B). In contrast, binding of THG1 to the smallest protein variant TSC22D1.2^{86aa} could not be detected. TSC22D1.2^{86aa} partially lacks the TSC22 box, which might reduce the affinity for THG1. Thus, THG1 forms heterodimers with both TSC22D1.1 and TSC22D1.2 protein variants, but as TSC22D1.1 is suppressed once OIS is established (Figure 5C), these results point to a predominant role for THG1–TSC22D1.2 complexes regulating OIS. Further corroborating this idea, ectopic expression of THG1 effectively abrogated proliferative arrest induced by BRAF^{E600} (Figure 7C and D).

Discussion

In this study, we found a causal relationship between transcriptional activation of *TSC22D1.2* and the establishment of OIS, a failsafe mechanism that restricts the expansion of incipient tumour cells. Interestingly, the short *TSC22D1* transcript (*TSC22D1.2*) was selectively and strongly induced by oncogenic stress, while the expression levels of the larger messenger (*TSC22D1.1*) remained unchanged. This differential regulation prompted us to examine their respective roles in OIS. Specific depletion of the small TSC22D1.2 protein variants was sufficient to abrogate BRAF^{E600}-induced senescence. While the expression level of the large *TSC22D1.1* transcript was not regulated during OIS, its protein product

Figure 5 Selective induction of the *TSC22D1.2* transcript is linked to OIS. (A) Scheme indicating the two transcripts, *TSC22D1.1* and *TSC22D1.2*, expressed from the *TSC22D1* locus. The known protein-coding transcripts have four exons, from which only the third and fourth are used in both transcripts. Our data suggest that the *TSC22D1.1* transcript encodes the TSC22D1.1 protein and that the *TSC22D1.2* transcript encodes three proteins, TSC22D1.2 (144, 134 and 86 aa), by differential use of translation initiation sites. All TSC22D1 protein variants, but TSC22D1.2^{86aa}, harbour the TSC box, a region that is highly conserved in all TSC family members (indicated in fair blue), and a leucine zipper (indicated in dark grey). (B) qRT-PCR of Tig3(et)-16i HDF with and without BRAF^{E600}. Samples were taken 10 days after infection and the transcript levels of *TSC22D1.2* and *TSC22D1.1* determined using specific primers. Transcript levels were standardized to those of proliferating control cells. Mean and s.d. of three independent experiments are shown. (C) Immunoblot with the indicated antibodies on lysates from the cells described in (B). Samples were taken 6 and 10 days after transduction with BRAF^{E600}. Arrowheads point to the TSC22D1 protein variants. β-Actin serves as a loading control. (D) HDF (Tig3(et)-16i) senescent and proliferating control cells were treated 9 days after transduction with the indicated constructs with the proteasome inhibitor MG132 (16 h at 10 μM). TSC22D1.1 and BRAF levels were determined by immunoblotting. β-Actin serves as loading control. (E) Scheme showing the *TSC22D1* sequences targeted by shRNAs. Whereas sh-TSC22(1) and (2) target all *TSC22D1* transcripts, sh-TSC22(3) specifically targets *TSC22D1.2* and sh-TSC22(4) *TSC22D1.1* only. (F) HDF were transduced with the hairpins described in (E) and subsequently transduced with BRAF^{E600}-encoding or empty vector (control). Protein levels were assessed 8 days after oncogene exposure by immunoblotting. β-Actin serves as loading control. (G) Cell proliferation assay of the experiment described in (E). Cells were stained 10 days after exposure to BRAF^{E600}.

was lost upon the establishment of OIS. Restoration of its expression abolished OIS, similarly to what was observed for depletion of the small protein products encoded by the *TSC22D1.2* transcript. These results, taken together, indicate that the small and large protein variants encoded by the *TSC22D1* gene exert opposite functions in the control of OIS,

with the small products being essential for the execution of the senescence program.

We found that the *TSC22D1.2* transcript can code, by alternative start codon usage, for three small polypeptides, two of which have been described previously (Shibanuma *et al*, 1992; Fiol *et al*, 2007). We show that, in contrast to the

Figure 6 Ectopic expression of TSC22D1^{1073aa} overrides OIS. (A) Cell proliferation assay of HDF (Tig3(et)-16i) transduced with a vector driving expressing TSC22D1.1 or TSC22D1.2a in the presence or absence of BRAF^{E600}, as indicated. Cells were fixed and stained 10 days after transduction with BRAF^{E600}. (B) In parallel to (A), samples were collected for immunoblotting to determine TSC22D1.1 levels. β-Actin serves as loading control.

Figure 7 TSC22D1 regulation of OIS involves its dimerization partner THG1. (A) HDF (Tig3(et)-16i) were transduced with lentivirus expressing the indicated genes or shRNAs. THG1 and BRAF levels were determined by immunoblotting 10 days after transduction with BRAF. β-Actin serves as loading control. (B) HDF were transduced with BRAF^{E600}-expression vector or control vector. Eight days after transduction, cells were harvested, lysed and subjected to immunoprecipitation (IP) with THG1-specific antibody. Immunoprecipitates were resolved and immunoblotting was performed with TSC22-specific antibody detecting all TSC family members including THG1 (open triangle). An antibody of the same species and immunoglobulin serve as controls. HC indicates the heavy chain, LC indicates the light chain of immunoglobulins. The image showing the input control was exposed for 2 min, the IP blot for 20 s. (C) Cell proliferation assay of HDF (Tig3(et)-16i) transduced with the indicated constructs. Cells were fixed and stained 10 days after transduction with BRAF^{E600}. (D) In parallel to (C), cells were harvested and analysed for THG1 protein levels by immunoblotting.

shortest form (86 aa), the 134-aa and 144-aa variants efficiently interact with THG1, a TSC22 family member. THG1 expression itself was not regulated by oncogenic stress. However, its ectopic expression was able to override OIS. Thus, OIS could be abrogated in three ways: by (i) depletion of the small TSC22D1.2 protein variants, (ii) ectopic expression of (the large) TSC22D1.1 protein and (iii) ectopic expression of the TSC22D1 dimerization partner THG1 (note that THG1 might form also homodimers). In aggregate, these observations suggest a model in which a complex between TSC22D1.1 and THG1 is compatible with prolifera-

tion (Figure 8). A simultaneous increase in the levels of TSC22D1.2 proteins (by indirect C/EBPβ-dependent transcriptional induction) in response to oncogenic signalling and decrease in TSC22D1.1 expression (by proteasomal degradation) forces a shift in protein complex stoichiometry: in the absence of TSC22D1.1, the small TSC22D1.2 proteins can engage in interactions with THG1, enforcing cellular senescence. In turn, when the ratio between TSC22D1.1 and TSC22D1.2 increases, the first can interact with THG1, allowing for OIS bypass and cell proliferation to occur. Although we show that TSC22D1.1 and TSC22D1.2 form

Figure 8 Regulation of TSC22 family members in OIS. Our results suggest a model in which TSC22D1.1 and THG1 form heterodimers in proliferating HDF or melanocytes. Upon BRAF^{E600} expression, TSC22D1.2 variants are strongly induced and compete with TSC22D1.1 (whose levels decline by proteasomal degradation) for binding to THG1, resulting in displacement of the latter and induction of OIS. OIS can be abrogated by the downregulation of TSC22D1.2 or the ectopic expression of either THG1 or TSC22D1.1. The latter interacts with THG1, allowing for OIS abrogation. Since TSC22D1.1 levels are very low in OIS, exogenous THG1 abrogates cell-cycle arrest most likely through homodimerization.

heterodimers with THG1, we cannot exclude the formation of functional THG1 homodimers, too.

Recently, antagonistic functions of the human TSC22D1 variants have been observed when they were introduced into *Drosophila* (Gluderer *et al*, 2010). Bun, the *Drosophila* homologue of the mammalian TSC22 family members, was replaced by either the human TSC22D1.1 or TSC22D1.2 variant. Consistently, in a lethality rescue experiment, TSC22D1.1 was able to substitute for BunA (the homologue of the long form), whereas TSC22D1.2 was not. Similar to human TSC22D1, Bun is encoded by several transcripts. Two

short variants, BunB and BunC, contain (similar to human TSC22D1.2) mainly the TSC box and the leucine zipper domain. In an earlier study, it was shown that BunB and BunC could interfere with the function of BunA, suggesting an antagonistic role of the long and small forms (Gluderer *et al*, 2008). Our findings lend strong support for this opposing function, arguing that the regulation and functional antagonism of human TSC22D1 variants are naturally exploited in the regulation of cell proliferation, particularly OIS. Our finding that TSC22D1.2 transcript levels are induced in OIS while TSC22D1.1 levels remain unchanged is consistent with the observation that TSC22D1.2 has transcription-regulatory elements that are distinct from TSC22D1.1 (Uchida *et al*, 2003).

TSC22D1 was initially identified as a TGF β -induced gene in murine osteoblast cells (Shibanuma *et al*, 1992). We could recapitulate this observation in TGF β -sensitive human HaCaT keratinocytes (Supplementary Figure S6). Interestingly, depletion of TSC22D1 almost completely abolished the sharp induction by TGF β of one of its established downstream effectors, *p15INK4B*. Furthermore, TSC22D1 was highly induced by oncogenic RAS (Supplementary Figure S7). Taken together, these observations raise the intriguing possibility that TSC22D1.2 has a common role in senescence and cytostatic signalling.

We show that TSC22D1.2 transcription is under strict control of the transcription factor C/EBP β . We and others have previously demonstrated that C/EBP β fulfills a critical role downstream of the RAS-BRAF-MEK-ERK cascade in OIS (Sebastian *et al*, 2005; Acosta *et al*, 2008; Kuilman *et al*, 2008; Atwood and Sealy, 2009). Consistent with this, C/EBP β depletion resulted in loss of TSC22D1.2 expression, indicating that the latter serves as a critical target for the first in OIS signalling. The exact relationship between C/EBP β and TSC22D1 remains to be resolved, however. Although there are two potential binding sites in the TSC22D1 promoter for C/EBP β , ChIP experiments failed to provide convincing experimental evidence for such a direct regulation. This notwithstanding, two-sample correlation plotting revealed that TSC22D1 proteins account for regulating a substantial proportion of C/EBP β target genes. These include interleukins *IL1 β* , *IL6* and *IL8*, as well as *CDKN2B* (encoding p15^{INK4B}). As we have previously demonstrated that these proteins correspond to factors that are critical for the implementation of the OIS program (Michaloglou *et al*, 2005; Kuilman *et al*, 2008), these results indicate that TSC22D1 controls OIS at least in part by regulating a significant portion of the inflammatory transcriptome, while also impacting on a specific cell-cycle inhibitor.

Finally, given that our findings in two HDF strains were confirmed in human melanocytes, the antagonistic interplay of TSC22D1 variants might also be involved in OIS of human nevi. Despite having oncogenic BRAF^{E600} mutations, these lesions arrest in a benign state and display markers of senescence. Infrequently, this failsafe mechanism is abrogated, conceivably as a function of the acquisition of secondary mutations. Loss of TSC22D1.2 may correspond to such a phenomenon. Consistent with this, we found that restoration of TSC22D1.2 expression induced cell-cycle arrest in a subset of melanoma cell lines that express low levels of TSC22D1.2 protein (data not shown). Although certainly not immediately applicable in this setting, restoration of senescence

in vivo has been shown recently in several models, including PTEN in prostate cancer (Chen *et al*, 2005, 2009). Also in other settings, like CDK2 inhibition of Myc-driven lymphomas (Campaner *et al*, 2010), Myc inactivation in T-cell lymphomas (van Riggelen *et al*, 2010) and CDK4 inhibition in KRAS-driven non-small cell lung carcinoma (Puyol *et al*, 2010), reactivation of the senescence response has been proposed to be therapeutically beneficial. Regarding a putative role for TSC22D1 in melanoma progression, it will be interesting to determine the expression levels of TSC22D1.2 and TSC22D1.1 in human nevi and malignant melanomas (and other cancers, including those of the prostate, in which TSC22D1 has been proposed to have a role). However, currently available antibodies cannot discriminate the small variants of TSC22D1 from its large variant. Nonetheless, the results presented here argue for further in-depth studies on the role of TSC22D1 as a candidate tumour suppressor, with its variant proteins exerting antagonistic functions.

Materials and methods

Cell culture, cell lines, cell proliferation assay

Melanocytes were propagated in 254CF medium in the presence of 0.2 mM CaCl₂ and melanocyte growth supplement (Cascade Biologicals). HDF line Tig3 expressing the ectopic receptor and hTERT (Tig3(et)), its derivative expressing sh-p16^{INK4A} (Tig3(et)-16i), and HaCaT cells, were maintained in DMEM, supplemented with 9% fetal bovine serum (Greiner, Bio-One), 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (GIBCO). The HDF cell line IMR90, expressing the ectopic receptor, shRNA for p16^{INK4A} and hTERT, was cultured in MEM + Earle's salts (GIBCO) containing the same supplement as the DMEM.

Cells were infected with shRNA-encoding or protein-coding retro- or lentivirus, selected pharmacologically (puromycin or blasticidin) and subsequently infected with BRAF^{E600}-encoding or control virus. After 6 days of selection, HDF and melanocytes were seeded for cell proliferation assays into a six-well plate of 6 cm plate (2 × 10⁵ or 4 × 10⁵ cells) and maintained in selection medium. Staining and fixation was performed 10 to 15 days after the last infection and plates were stained with crystal violet or Coomassie blue. BrdU labelling was carried out for 3 h followed by fixation. Incorporated BrdU was detected by immunostaining and FACS analysis. Experiments are represented as mean with s.d.

For stimulation with TGFβ (R&D), Tig3(et)-16i and HaCaT cells were treated with 200 pM of the cytokine, for 7 days. Proteasomal inhibition was achieved by treating Tig3(et)-16i HDFs for 16 h with 10 μM of the inhibitor MG132 (carbobenzoyl-L-leucyl-L-leucyl-L-leucinal from Sigma).

Senescence-associated β-galactosidase was stained using the 'Senescence β-Galactosidase Staining Kit' from Cell Signaling at pH 6. Images of cell proliferation assays and cells reflect representative results of several independent experiments.

Plasmids

pMSCV-blast-BRAF^{E600}, pMSCV-blast, pMSCV-blast-KRAS^{V12}, pBABE-puro-BRAF^{E600}, pBABE-puro, pLZRS-IRES-zeo-Cdc42^{V12} were previously described (Kuilman *et al*, 2008). For the over-expression of TSC22, the ORFs of TSC22D1.2 and TSC22D1.1, which were derived from cDNA of a normal human prostate tissue sample (Rentsch *et al*, 2006), were PCR amplified and cloned into pBABE-puro. The optimal Kozak sequence (TSC22D1.2a) or the genomic Kozak sequence (TSC22D1.2b, c and TSC22D1.1) was added using the corresponding primers. To construct TSC22D1.2c, the second ATG encoded by the ORF was mutated into TTG using PCR amplification. This results in an amino-acid change from methionine to leucine. Both amino acids are non-polar and this substitution is therefore expected to have no major effect on the protein function.

shRNAs

Sequence of shRNAs cloned into pRetroSuper (puro) (Brummelkamp *et al*, 2002) or KH1 (kind gift from M Soengas):

sh-C/EBPβ#1 see Kuilman *et al* (2008)
sh-TSC22D1#1: GTAAATCCTTGTCTACTGA;
sh-TSC22D1#2: GAGCAAATCAAAGAAGTA;
sh-TSC22D1.2#3 CGCTTCTGTGAGACTTGAT;
sh-TSC22D1.1#4 TGGTTCAAAGTGTAGTCAAC.

Microarray gene-expression profiling

Cells were infected with shRNA-encoding retrovirus briefly selected with puromycin and subsequently infected with BRAF^{E600}-encoding or control virus. After 8 days under constant selection for BRAF^{E600} (with blasticidine), cells were used for RNA extraction. Total RNA from two independent experiments was isolated, purified and analysed on Human Illumina BeadArray V3. Data were normalized using the Affymetrix model for background correction (robust spline normalization). The data were further processed using the 'lumi', a pipeline for the processing of Illumina microarray data, see Du *et al* (2008).

Accession numbers

Microarray data have been deposited in the ArrayExpress repository under accession numbers E-NCMF-12 and E-MTAB-441.

Quantitative real-time RT-PCR

Primer sets used were as follows:

IL6, IL1β, IL8, C/EBPβ, HPRT1 (standard) and RPL13 (standard) primer sequence (Kuilman *et al*, 2008).

TSC22D1.1: 5'-TGGGAGTAGCAGCAGCCTAGCA-3' and 5'-CCAGAGGAGCTCTCATCTCGCC-3'; TSC22D1.2: 5'-GGACTGAAACGCGCTTCTGTGA-3' and 5'-TTGTCAATAGTACCACACTTGC-3'

Total RNA was DNase treated with RQ1 RNase-Free DNase (Promega). Reverse transcription was performed with SuperScript II First Strand Kit (Invitrogen). qRT-PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems) on an ABI PRISM 7700 Sequence detection System. RPL13 and HPRT1 were used as control. For analysis, the ΔT method was applied.

Antibodies

Antibodies used for immunoblotting were β-actin (AC-74; A5316; Sigma), BRAF (sc-5284; Santa Cruz), C/EBPβ (sc-150; Santa Cruz), TSC22D1-specific antibodies (detecting all TSC22D1 proteins) R2 (Rentsch *et al*, 2006), TSC22 family specific (detecting all TSC22 family members including TSC22D1, D2, D3 and D4 (THG1)) R1 (Rentsch *et al*, 2006), THG1 (HPA006757; Sigma), CDK4 (sc-260; Santa Cruz), p44/42 MAPK (Erk1/2) (9102; Cell Signaling), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ((E10, 9106; Cell Signaling), MEK1/2 (L38C12; 4694; Cell Signaling), phospho-MEK1/2 (Ser217/221) ((41G9; 9154; Cell Signaling), p53 sc-126 (Santa Cruz), p15^{INK4B} (sc612; Santa Cruz).

For immunoprecipitation, precleared lysates in lysis buffer (1% Triton, 0.5% NP-40, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4) from HDF with or without BRAF^{E600} were incubated with THG1 antibody (HPA006757; Sigma) and immunoprecipitated using Protein A sepharose beads (GE Healthcare).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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