In response to our recent report on the Brdm2 mouse model for p63 function, Mikkola et al. raise concerns on three of our statements: 1) They maintain their initial claim that no functional p63 protein of any kind is synthesized in mice homozygous for the Brdm2 allele; 2) they report the occurrence of spontaneous wild-type allelic reversions in these mice, leading to patches of completely normal epidermis, and propose that these normal reverted skin patches are in fact the multilayered epithelia we described; 3) they insist that the Brdm2 allele is phenotypically indistinguishable from a global p63 knockout.

We started working with Brdm2 mice (which we received from A. Mills about 3 years ago) as a source of p63 KO mice. Extensive molecular and protein analysis of 26 different p63 Brdm2/Brdm2 embryos from multiple generations and multiple litters ranging from day E13 to P1 consistently revealed expression of aberrant truncated p63 variants, not confined to random epidermal patches but widespread, and associated with a transient phenotype consistent with residual functions of the targeted allele, as detailed below. We further showed that, in reporter assays, this Brdm2-derived p63 can both activate (as TAp63) and repress (as ΔNp63) a p53-responsive promoter. We therefore cannot help but maintain the notion that Brdm2 represents a functional aberrant hypomorphic allele but not a null allele.

Spontaneous reversion of the Brdm2 allele to restore wild type p63?

Mikkola et al. imply that the most likely explanation for all our observations is that we unknowingly fell victim of numerous mosaic reversion events in which the wild type p63 allele is recreated through spontaneous homologous recombination in individual cells, leading to isolated patches of completely normal wild type skin (see Figure 4 of Mikkola et al.). They further believe that the reason why we were unable to detect reverted alpha (and beta/gamma) p63 transcripts was that we missed the alpha terminus because we analyzed mRNA isolated from whole embryos rather than from isolated skin.

However, we believe that while mosaic reversion can occasionally occur, this is not what generated the predominant Brdm2 phenotype that we analyzed. Instead, this phenotype is due to consistent widespread expression of truncated p63 proteins from the Brdm2 allele. The reasons are given below.
In contrast to classic KO constructions that target more upstream exons of the genes to be silenced, the p63 Brdm2 allele is unusual because the targeting vector inserted late, after Exon 10, leaving most of the p63 gene intact. Thus, nothing prevents the expression of a p63γ-like protein (which only misses a small 37 aa tail encoded by Exon 10'). The allele was generated by the so-called gap-repair mechanism via insertional mutagenesis from a pre-existing vector library, resulting in a duplication of a segment of the p63 gene and the retention of a Hprt minigene.  This method of allele targeting, although quick, bears several risks. One is indeed instability of the targeted allele, which apparently is occurring but had not been previously acknowledged. Mikkola et al.2 (Figure 4) now report that they “routinely observe such reversion events in Brdm2 embryos of all developmental stages”. Of note, however, in 3 years of breeding, we never observed the strong phenotypic reversion indicated by macroscopically visible skin patches in our late Brdm2 embryos. Thus, it seems that the mosaic reversion they now describe is rare enough that we did not see it in our large colony. On the other hand, as for a possible germline reversion, the phenotype should then be heterozygous which is phenotypically normal, and can therefore be excluded.

The second problem present in the Brdm2 allele is the possibility of Hprt fusion proteins derived from the Hprt minigene of the targeting vector. It is well known that Exons 3-9 of Hprt splice into upstream exons of the targeted gene, forming chimeric transcripts and proteins expressed from the endogenous promoter. In fact, this trap has been deliberately exploited to generate truncated Brca2 and Top3β mice.4 We showed that Hprt Ex3-9 fusions with p63 Ex1-10 consistently occur in all examined Brdm2 mice.

Wild-type reversion would recreate the alpha isoform and then be the dominant product again, making it easy to detect. Another important point against our observation simply being an overlooked wild-type reversion is that we do not see p63α in Brdm2/Brdm2 mice (BB), neither on the transcript nor on the protein level. We specifically analyzed side-by-side p63 protein expression in snout and oral epithelium from E15 WT, BB littermates and McKeon p63KO embryos 5, because at E15, nasal vestibulum and oral epithelium of all analyzed BB embryos showed extensive squamous epithelium. Of note, ΔNp63α was readily detectable in WT but missing in BB and p63KO snout/oral epithelium (see Figure 1a; 4A4 immunoblot with equal total protein loading, reprinted from Supplementary Figure 2 of Wolff et al.1). On the other hand, the weaker band of γ-like ΔNp63 (3'-10) in BB tissue (Figure 1a) is consistent with the situation present in p73, in that transcriptionally active isoforms of p73 are generally less stable than inactive isoforms.6 Thus, it is very possible that p63 isoforms lacking the long α/β tail (as in the Brdm2 products) are also less stable than alpha isoforms. Their lower abundance, however, does by no means automatically imply negligible activity. This is clearly exemplified in the case of p73, since endogenous p73 protein levels are much lower than those of p63, yet p73 knockout cells and mice exhibit multiple phenotypes.

In contrast to our studies, Mikkola et al.2 were unable to detect p63 by immunoblot in BB embryos. However, failure to detect a protein cannot be taken as evidence that such a protein does not exist. Also, while the immunoblot in Fig. 2 of Mikkola et al.2 does not show a signal below 43 kDa, it is unclear what antibody and whether sufficient detection sensitivity was used. Surprisingly, Mikkola et al.2 mark bands on this blot as non-specific without
proving that they are not derived from p63. Non-specific bands can only be declared as such when still present in a global p63KO sample, which is not provided in this blot. Thus, their Figure 2 can by no means be taken as conclusive evidence for the absence of any p63 isoform in BB mice. It only shows the absence of ΔNp63α in these mice, in perfect agreement with our finding.

**Expression of functional p63 variants from the Brdm2 allele**

The p63-HPRT fusion transcript we saw in whole embryos would not be possible after germline reversion. It could still be detectable in mosaic reversion, but - importantly - in addition to a proper p63α transcript that we do not see. Instead, we see expression of truncated p63 Ex1-10 in all embryos. Ex1-10 can be theoretically predicted due to a stop codon in Intron 10, which we confirmed by RACE. On the other hand, Ex4-11 (bridging the vector) as well as Ex12-13 and Ex13-14 alpha transcripts were not detectable. In further support of our conclusion, we repeated RT-PCR analysis of isolated skin from E17 embryos and again find strong p63Ex9-Hprt Ex7 fusion transcripts in BB and B/+ embryos, but not in WT embryos (Figure 1b). This indicates active expression of the Brdm2 allele in skin (which at this stage consists of epidermal remnants and dermis). As suggested by Aberdam and Mantovani7, we cannot exclude the possibility that our mixed background (C57Bl6/129, 50:50) did not reinforce expression and stability of the truncated isoforms. At any rate, whether or not this plays a role, it allowed us to uncover the potential of p63γ-like isoforms and underscored the importance of α/β isoforms in maintaining mature durable skin. The latter notion constitutes the main scientific conclusion of our study.

The nuclear p63 expression we saw by immunostaining with mono- and polyclonal p63-specific antibodies was not only detectable in microscopic skin patches (this would support the mosaic model), but also as contiguous circumferential squamous epithelium in some E15 embryos. Most importantly, p63 was consistently expressed in internal epithelial layers of the oral cavity, tongue, nasal vestibulum, esophagus, forestomach, bladder, pituitary, and germ cells in ovary where p63 staining was not mosaic but rather uniform and extensive.

**Phenotypic changes due to the Brdm2 allele**

Mikkola et al.2 assert that the surface epithelium of Brdm2/Brdm2 mice remains a single-layered ectoderm throughout gestation, expressing only ectodermal markers K8 and K18. They further assert that at no stage during development does it express markers of more advanced epithelial development and differentiation, such as K14 and Perp.

In contrast, in our hands the p63 protein made in Brdm2 embryos was associated with initiation of skin (positive for K14 and Perp) and appendage development at E15. Importantly, however, this epidermal initiation was of a very transient and fragile nature and had largely disintegrated again by E18, except for some remnants. The fact that the epidermis was disintegrating by E18 and thus definitely not normal skin further excludes reversion as its basis. Rather, this aberrant epidermis was most likely the product of truncated Brdm2 proteins. Since residual multilayered squamous epithelia were mostly confined to the nasal and oral cavities that are mechanically protected from abrasion in a crowded multilitter uterus, we propose that the Brdm2 allele allows a longer maintenance of
such aberrant epithelia in those areas that are under less mechanical stress. Likely, the alpha and beta isoforms of p63 are required for maturation and to ensure proper cell-cell adhesion of epidermal cells, explaining the transient nature of epidermal formation that we observed in Brdm2/Brdm2 mice. In contrast, the inner epithelia had more consistent p63 expression and were less prone to disintegration. In fact, the pituitary looked completely normal at E18. Thus, we provide several lines of evidence that in sum convincingly demonstrate the function of this aberrant Brdm2 allele.

To clarify, at no time did we claim that Brdm2/Brdm2 mice have a normal skin phenotype. Even the title of our previous report implies that normal epidermis requires full-length p63, including the α/β encoding exons. However, we do observe a phenotype that casts doubts on a complete loss of function.

The lack of α/β isoforms may abolish the majority of ΔNp63 functions. In contrast, with respect to transcriptionally active TAp63 isoforms, maintenance of a γ-like allele may compromise p63 function to a lesser extent. TAp63α and β display far higher activity when lacking the C-terminal region due to removal of the intramolecular repression by the TID domain (transactivation-inhibitory domain). Thus, the Brdm2 allele may even enhance TAp63-like functions, rather than abolish it. In support of this notion, we observed that expression of a single Brdm2 allele bestows protection from lymphoma development on Eμ-Myc mice (Figure 1c). The Eμ-Myc model is centrally dependent on the p53 tumor suppressor pathway. Thus, protection is readily explained by an additional p53-like allele that TAp63(1-10) constitutes, in addition to the 2 endogenous p53 alleles present in these mice. These data are in complete agreement with Keyes et al.10, albeit with a different interpretation.

**Future research strategies**

Taken together, current knowledge of the Brdm2 model can be best summarized by saying that Brdm2 represents an aberrant hypomorphic allele of p63, but should not be considered the equivalent of a complete knockout. Some p63 functions nonetheless seem strongly impaired in p63Brdm2, and in these cases, the conclusions drawn from studies on Brdm2/Brdm2 mice remain valid. On the other hand, other functions, especially regarding TAp63, may not be impaired or even exaggerated in Brdm2/Brdm2 mice, and studies of these functions indeed require careful re-interpretation. In addition, although apparently rare, the Brdm2 allele carries some risk of instability due to reversion.2 A global p63 knockout seems to be the preferable choice for a model system. Also, the field has moved on and by now, additional mouse models of p63 ablation are available including conditional isoform-specific knockouts which should further clarify our present understanding of p63 function.

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Figure 1a. Brdm2 mice do not make δ63α protein. Reprint of Supplemental Figure 2 of Wolff et al., 2009.1 Side by side analysis of δ63 expression of tissue lysates from the snout and mouth region of WT, Brdm2/Brdm2 (BB) littermates and McKeon p63KO embryos at day E15 (lanes 3-6, 200 μg lysate each, 4A4 antibody). At E15, nasal vestibulum and oral epithelium of all analyzed BB embryos showed extensive squamous epithelium by histology. Note that ΔNδ63α (blue dot) is readily detectable in WT but missing in BB and p63KO snout/oral epithelium. Conversely, BB lysate contains a unique and reproducible...
band that aligns with ΔNp63(3′-10) (red dot) which is absent in WT and p63KO embryos. Lanes 1-3, H1299 cells transfected with the indicated p63 expression constructs for controls.

**Figure 1b. Strong p63Ex9-Hprt Ex7 fusion transcripts are detected in BB and B/+ embryos, but not in WT embryos.** RT-PCR analysis of isolated skin from E17 embryos of the indicated genotypes.

**Figure 1c. The presence of a single Brdm2 allele bestows protection from lymphoma development.** Kaplan-Meier survival curves of Eμ-Myc mice of the indicated genotypes. All mice were of mixed C57Bl6/129, 75:25 background and littermate controlled. Cox log-rank analysis, p-values are indicated.