Cell Death and Differentiation (2006) 13, 1614–1618

www.nature.com/cdd

Letter to the Editor

Adult mice lacking the p53/p63 target gene *Perp* are not predisposed to spontaneous tumorigenesis but display features of ectodermal dysplasia syndromes

Cell Death and Differentiation (2006) **13,** 1614–1618. doi:10.1038/sj.cdd.4401871; published online 17 February 2006

Dear Editor,

Members of the p53 family of transcription factors, including p63 and p73, play distinct roles in tumor suppression and development. p53 plays a prominent role in tumor suppression, as indicated by the high frequency of *p53* mutations found in human cancer and by the fact that *p53*-deficient mice are highly cancer prone.[1] In contrast, while some evidence indicates a role for p63 and p73 in tumor suppression,[2] the best characterized functions of the p63 and p73 proteins are in development. *p73*−/− mice are prone to lethal infections, as well as abnormalities in brain development and pheromone sensing, while *p63*−/− mice fail to develop skin, oral epithelia, or other ectoderm-derived appendages.[1] Mutations in *p63* also have been identified in multiple human ectodermal dysplasia syndromes, in which patients display abnormal development of ectoderm-derived tissues, including the skin, teeth, hair, and nails, along with other symptoms.[1] Recent studies suggest further that loss of p63 in the stratified epithelia of adult mice results in cellular senescence and premature organismal aging.[3] Together these findings underscore the pivotal role of p63 in the proper development and function of ectoderm-derived tissues. However, the mechanisms by which p63 acts remain largely unknown, as few direct target genes mediating p63 function in epithelia have been identified. Like the transcription factors that direct their expression, p53/p63 target genes might also be candidates for mutation in diseases affecting tissue homeostasis, aging, or tumor suppression in the adult organism.

Perp is one of only a few target genes known to be activated by both p53 and p63. *Perp* is an apoptosis-associated p53 target gene essential for DNA damage-induced, p53-dependent cell death in specific cellular contexts.[4] In contrast, *Perp* expression in unstressed stratified epithelial cells depends on p63, and *Perp*−/− mice die within 10 days after birth due to compromised adhesion and blistering in the oral mucosa and skin.[5] In newborn epithelia, Perp functions in the desmosome, a multiprotein complex required for normal cell–cell adhesion in stratified epithelia. Consistent with a dual role for Perp, experiments in zebrafish have shown that Perp participates in both UV-induced, p53-dependent apoptosis and proper development of the skin and pectoral fins.[6] In both of these model organisms, loss of Perp has significant deleterious effects in the developing animal, but the more long-term effects of this absence have yet to be determined.

Perp loss, and consequent defects in apoptosis and/or adhesion, might be expected to alter adult tissue homeostasis and disrupt normal tissue function. For example, *Perp*-deficiency might impair the tumor suppressive function of both the p53 and p63 pathways, resulting in an enhanced spontaneous predisposition to cancer. Alternatively, *Perp* nullizygosity in adult mice might result in defects in the skin and other ectodermal derivatives that depend on p63 for normal development and maintenance. To define the role of Perp in adult tissue function, we examined aging *Perp*+/− and *Perp*−/− mice for tumor development and epithelial abnormalities.

We first assessed whether *Perp* heterozygosity predisposes mice to cancer, as in the case of classic tumor suppressor genes. Cohorts of F1 *Perp* heterozygous mice and wild-type littermates were generated on a 129/Sv;C57Bl/6 mixed genetic background to avoid strain-specific tumor predispositions. A group of 129/Sv;C57BL/6 F1 *p53* heterozygous mice was generated in an analogous fashion as a positive control for accelerated tumorigenesis. All cohorts were aged until they displayed morbidity (up to three years), monitored regularly for tumor development, and surveyed by careful histological analysis. *p53* heterozygous mice exhibited the expected increased mortality in comparison to wild-type mice, with a median survival time of 18 months, consistent with previous findings.[6] Upon aging the wild-type and *Perp* heterozygous mouse cohorts, no statistically significant difference in survival was observed: both genotypes displayed a median survival time of 28.5 months, with a similar incidence of tumor development in both groups (Figure 1a). In total, 80% of the mice of each genotype developed at least one benign or malignant tumor before death, and 21% of each cohort developed multiple tumors. Furthermore, the spectrum of tumors observed in *Perp*+/− animals was largely similar to that observed in wild-type mice, with approximately 50% of mice developing lymphomas or histiocytic sarcomas, and the remaining mice developing a variety of other tumors, including hepatocellular carcinomas, lung adenocarcinomas and pheochromocytomas (Figure 1b). In general, tumors in *Perp*+/− mice displayed the characteristics typical of malignant tumors, including a high percentage of mitotic figures and nuclear pleiomorphism (data not shown), and were indistinguishable from the tumors observed in wild-type mice. We also examined a subset of these tumors for loss of heterozygosity

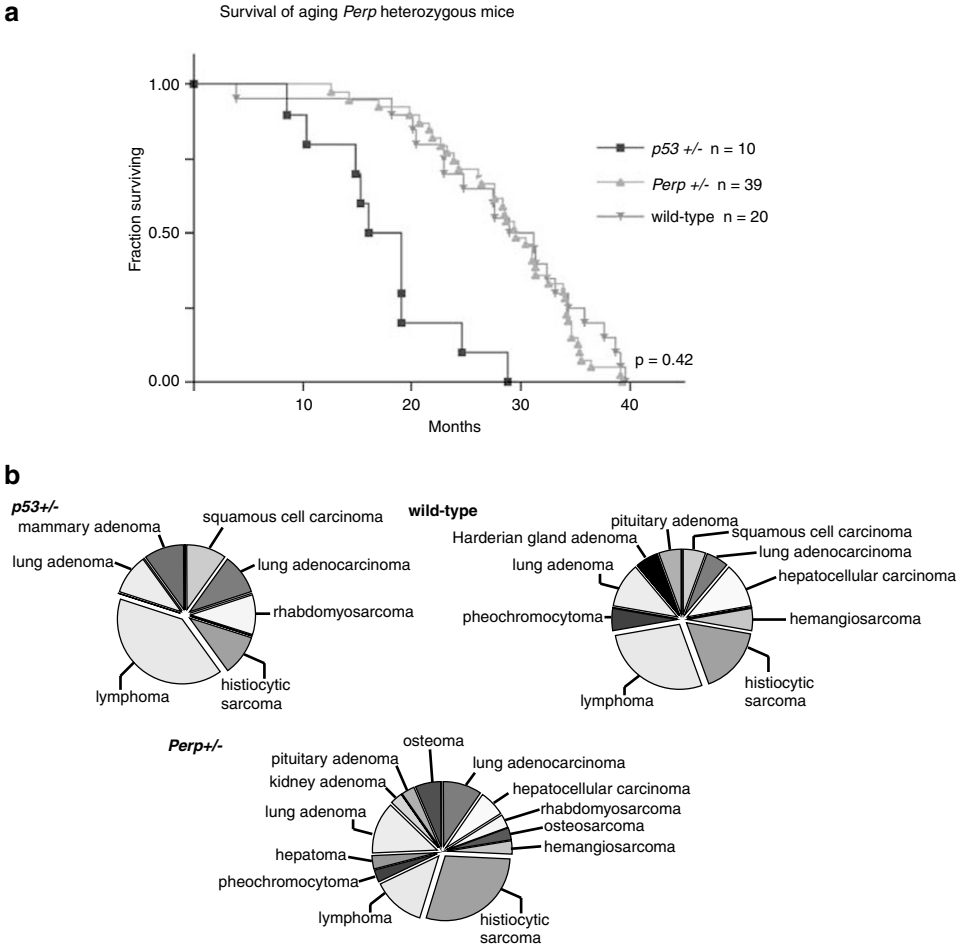

Figure 1 *Perp* + /– adults do not display accelerated tumor development. (**a**) While *p53* + /– adults develop tumors at a median age of approximately 18 months, both wild-type and *Perp* + /– mice survive to a median age of approximately 30 months, with no statistically significant difference in tumor-free survival by Kaplan–Meier analysis ($P = 0.42$, logrank χ^2 test). Graphing and statistical analysis were performed using GraphPad Prism. (**b**) The tumor spectra of *Perp* + /– and wild-type mice. The frequency of each tumor type, including both malignant and benign tumors, is indicated by genotype

(LOH) at the *Perp* locus by Southern blot analysis, and found no LOH in the 10 tumors examined (data not shown). Together, these findings indicate that *Perp* heterozygosity does not predispose mice to spontaneous tumorigenesis.

As the rate of LOH might be insufficient to reveal a predisposition of heterozygous mice to tumor development, we also aged *Perp*–/– mice to examine longevity and tumor development. Although *Perp*-deficiency induces completely penetrant lethality on a pure 129/Sv background within the first week of life, a small fraction (~5%) of *Perp*–/– mice survived to adulthood on a mixed 129/Sv;C57BL/6 background.[5] Upon aging these *Perp*–/– mice, we found that they exhibited a significantly decreased lifespan, with a median survival time of only 18 months compared to the survival time of 30 months observed in a wild-type cohort (Figure 2a). *Perp*–/– mice manifested a variety of symptoms prior to death, including decreased weight (31.25% of mice), inflamed skin or rashes (25%, Figure 2b), and fur with a patchy, disorganized or greasy appearance (43.75%, Figure 2b). Many mice also had swollen feet and toes, and in some cases the nails of these mice were blunted, broken, or absent (31.25%). No obvious tumors were detectable by eye, although a single tumor became apparent upon histological examination (discussed below). Two *Perp*–/– mice also exhibited partial paralysis.

The tissues affected in *Perp*–/– mice were reminiscent of those affected upon *p63* mutation. For example, *p63* + /– mice display hyperproliferation in the stratified epithelia, while mice deficient for *p63* in stratified epithelia show alopecia, lordokyphosis (curved spine), and decreased weight, suggesting premature aging. In addition, *p63* mutations in humans are linked to ectodermal dysplasia syndromes, in which the development or differentiation of the ectoderm and its derivatives is affected. The gross phenotypes in the skin, hair, and nails of *Perp*–/– adult mice suggested that the absence of Perp might result in defects resembling those seen in *p63*-deficient mice or in human diseases linked to compromised p63 function.

To examine the specific function of Perp in the adult organism, we performed a comprehensive histological analysis of all tissues from these mice. The abnormalities we identified were generally restricted to the stratified epithelia,

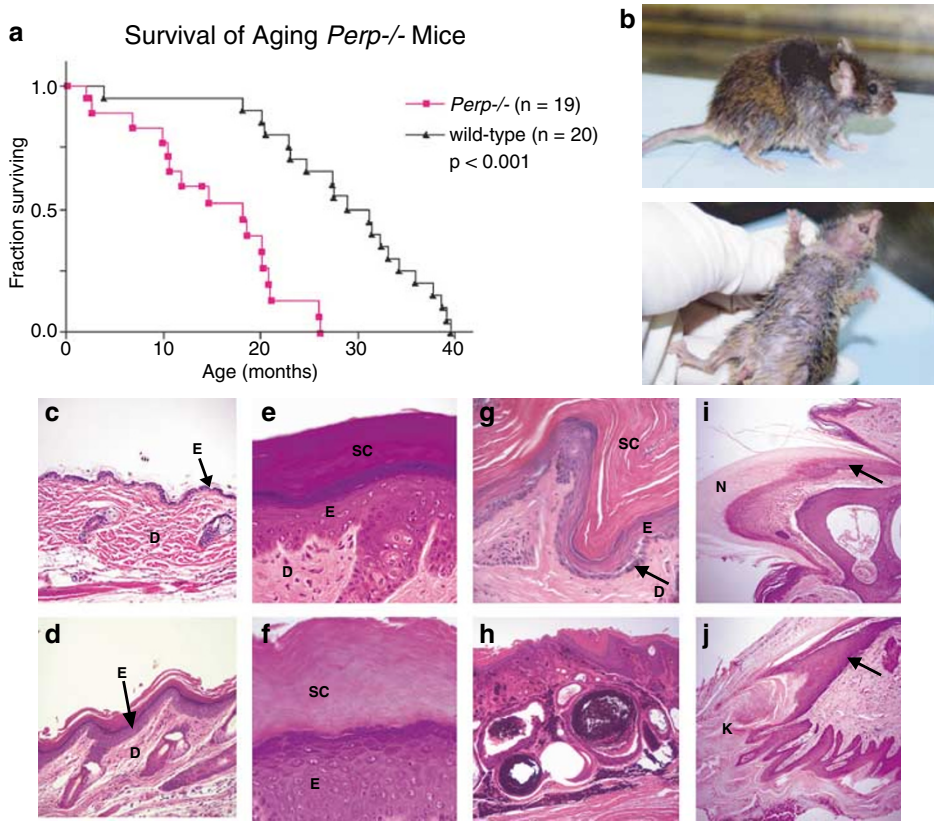

Figure 2 *Perp−/−* adult mice exhibit premature mortality and abnormalities in the stratified epithelia. (**a**) Kaplan–Meier analysis comparing aging mixed background *Perp−/−* mice, with a median survival of approximately 18 months, to a cohort of wild-type mice, with a median survival of 30 months ($P < 0.001$, logrank χ^2 test). (**b**) At the time of death, *Perp−/−* adults display gross abnormalities of the skin and fur. Mice have a greasy and disorganized coat (upper and lower images), swollen feet, and often develop a ventral rash or dermatitis (lower image). (**c–j**) Histological abnormalities observed in adult *Perp−/−* mice. All samples were prepared by fixation in neutral buffered formalin before processing, sectioning, and staining with hematoxylin and eosin. *Perp−/−* adults exhibit hyperplasia in the dorsal skin (**d**), with thicker epidermis (arrows) than age-matched wild-type adults (**c**). Epidermis and dermis are indicated by E and D, respectively. (**e–g**) While plantar skin (the sole of the foot) in wild-type mice is thicker than at other sites in the body (**e**), *Perp−/−* adults often exhibited extremely hyperproliferative and thickened epidermis on the feet (**f**) compared to controls. Many *Perp−/−* adults also developed hyperkeratosis (**g**) and some blistering on the soles of the feet (arrow, **g**). Both of these plantar epidermal phenotypes resemble human palmoplantar keratoderma and correspond to the swollen feet observed by gross pathology. E, epidermis; D, dermis; SC, stratum corneum of epidermis. (**h**) A single *Perp−/−* mouse also developed a melanotic hair follicle tumor on the dorsal skin of the foot. (**i,j**) *Perp−/−* adult nails develop abnormally. Sagittal sections of age-matched wild-type nail (**i**) show the typical organization of the squamous epithelium within the nail matrix (arrow) and the mature nail (indicated by N). (**j**) By contrast, the nail matrix epithelium of *Perp−/−* adults (arrows) is often profoundly disorganized and fails to produce a normal nail, instead giving rise to small 'nailettes' or disorganized keratinized layers (indicated by **k**), corresponding to the blunted or absent nails detected by gross pathology

including the skin, hair, and nails. Examination of the skin revealed multiple abnormalities. The dorsal skin of adult *Perp−/−* mice was abnormally thick, suggesting that adult *Perp−/−* skin, like that of *Perp−/−* newborns, is hyperproliferative (Figure 2c and d). Many of the *Perp−/−* mice (25%) also had infiltrating immune cells and tissue inflammation in the dorsal or snout skin, consistent with the frequent detection of severely inflamed skin by macroscopic analysis at the time of death. The skin on the feet (plantar skin) of multiple *Perp* null animals also appeared abnormal, with either extensive thickening of all epidermal layers (keratoderma, shown in Figure 2f) or an increased number of enucleated corneal layers (hyperkeratosis) with splitting between cells (68.75% mice, Figure 2g). These plantar skin phenotypes closely resemble human palmoplantar keratoderma, a condition linked to mutations in desmosomal proteins or components of the associated keratin cytoskeleton, in which the skin of the palms and soles is thickened and may exhibit blistering.[7] Keratoderma is also a feature of many ectodermal dysplasia syndromes.[8] Many of the mice with features of palmoplantar keratoderma also exhibited inflammation on the soles of the feet – a condition known as pododermatitis (25% of all mice, 36% of those with keratoderma). We did not observe these lesions in age- and background-matched mice, indicating that they are due specifically to *Perp* loss. These findings show that the absence of Perp in the adult epithelia results in severe skin abnormalities – keratoderma and dermatitis – that are seen in human ectodermal dysplasias and related syndromes.

In addition, five *Perp−/−* animals (31.25%), including the two animals that suffered from paralysis, had severe bacterial infections. Colonies of bacteria were visible in large abscesses in the jaw, ear, or spinal cord (data not shown), with the abscesses in the ear and spinal cord inducing paralysis. This increased susceptibility to infection and inflammation is possibly due to impaired barrier function in the skin of *Perp−/−* mice. As *Perp*-deficiency compromises desmosome

assembly, adhesion defects in *Perp−/−* adults are likely to reduce the integrity of the skin and the ability of the animal to avoid infection. Defective epithelial integrity resulting from desmosomal alterations has been observed in transgenic mice in which the composition of the desmosome is altered by ectopic expression of specific desmosomal cadherins in the skin.[7] The enhanced susceptibility of *Perp−/−* mice to infection also recalls human ectodermal dysplasias, in which patients may develop chronic infections in the ear, nose, and throat.[8] Although rampant infection was a clear cause of death in this subset of *Perp−/−* mice, the source of lethality in animals that did not succumb to infection remains to be determined. No obvious histological abnormalities were identified in other tissues, but we cannot exclude the possibility that subtle functional defects are present in other Perp-expressing tissues such as the heart.

In addition to skin abnormalities, *Perp−/−* adults also displayed defects in the development of ectodermal appendages, consistent with the gross abnormalities observed in the nails and hair. A subset of *Perp* null animals (31.25%) had significant alterations in the architecture of the squamous epithelium of the nails, resulting in a highly disordered structure. While the nail unit of wild-type mice displays the same general organization as the skin, with a sheet of basal, transit-amplifying cells giving rise to stratified layers of keratinized cells (Figure 2i), this structure was disrupted in *Perp−/−* mice. The nails of *Perp−/−* adults contained basal layers that appeared disorganized, with multiple small 'nailettes' developing rather than a coherent stratified structure (Figure 2j), and an absence of any visible nail plate in some mice. These phenotypes recall the dystrophic or malformed nails typical of some human conditions including ectodermal dysplasia and pachyonychia congenita, which is frequently associated with mutation of cytokeratins. Finally, the greasy and disorganized hair observed by gross pathology (Figure 2b), as well as the melanotic hair follicle tumor observed in one *Perp−/−* mouse (Figure 2h), suggests compromised function of the hair follicle, another ectodermal appendage. With the exception of the single hair follicle tumor, no tumors were evident in *Perp−/−* mice by histological examination, reiterating that loss of Perp alone, despite the extensive hyperproliferation observed in the skin, is not sufficient to initiate tumorigenesis within the limited lifespan of these mice. These data support our previous observations showing that Perp ablation in the skin does not enhance, and in fact decreases, the predisposition of mice to tumor development in a two-step carcinogenesis protocol, suggesting that Perp, and intact desmosome function, are required for tumor initiation in the skin.[9] Together, these findings highlight the important role for Perp in proper homeostasis and function of the ectoderm and its derivatives, rather than in suppression of tumor initiation.

The lack of spontaneous tumor predisposition in the *Perp*-deficient mice is consistent with studies showing that although *p53−/−* mice rapidly develop cancer at 100% frequency, mice lacking specific p53 target genes – including *p21*, *GADD45*, *bax, puma,* or *noxa* – are not clearly tumor-prone.[4,10,11] This distinction in tumor phenotypes suggests that p53 may carry out its tumor suppressive function through a network that includes many targets, and that multiple target genes must be lost to duplicate the *p53−/−* spontaneous tumor phenotype. Alternatively, the relevant p53 targets that contribute to its tumor suppressive effects *in vivo* may not yet be known. These data do not, however, exclude a function for Perp in tumor suppression in certain contexts, for example by limiting tumor progression or metastasis. In keeping with this idea, *Perp+/−* mice exhibited a slight increase in the propensity of tumors to metastasize (4/32 malignant tumors analyzed in *Perp+/−* mice *versus* 1/15 in wild-type mice and 0/10 in *p53+/−* mice), suggesting that diminished expression of Perp might decrease cell–cell adhesion and increase the invasiveness of cells within established tumors. Indeed, the human homolog of *Perp*, *THW*, is downregulated in metastatic melanoma when compared to primary tumors,[12] suggesting that although Perp loss is not sufficient for tumor initiation, subsequent loss of Perp may enhance metastatic potential. This notion is consistent with several studies showing that desmosomal components are downregulated during tumorigenesis, suggesting that compromised desmosomal adhesion may promote tumor metastasis.[13] In future, it will be of great interest to investigate Perp's contribution to tumorigenesis in later stage cancers.

The phenotype of *Perp−/−* mice is most obvious in ectodermal derivatives including the skin, nails, and hair, and the observed abnormalities are reminiscent of both the aging phenotype observed in *p63*-deficient mice and the ectodermal dysplasia seen in humans with *p63* mutation. Although the phenotype of *Perp−/−* adults resembles premature aging in some respects, including a predisposition to infection and reduced body weight, several other features of accelerated aging, such as lordokyphosis, hair graying, alopecia, decreased bone density, and decreased organ mass, were not observed in *Perp−/−* adults (data not shown). Therefore, *Perp−/−* mice do not seem simply to be exhibiting premature aging. Instead, the characteristics of adult *Perp−/−* mice resemble the ectodermal dysplasia syndromes associated with mutations in *p63* and the various diseases linked to compromised desmosome function, which also display features of ectodermal dysplasia. Ectodermal dysplasias comprise a large family of diseases in which development of the skin and other ectodermal appendages occurs aberrantly. In these diseases, at least two appendages among the hair, teeth, nails, and sweat glands are generally affected, and they are classified according to the particular appendages that are involved. In addition, abnormalities may be evident in other epithelia, including the cornea, oral mucosa, or the skin, particularly the lips, ears, palms and soles. Finally, affected structures may also include those that rely on epithelial-mesenchymal signaling for development, such as the palate or limbs.[1,8] Ectodermal dysplasias fall within the larger class of ectodermal dysplasia syndromes, which include diseases that have certain symptoms in common with ectodermal dysplasia, but are linked to mutations affecting differentiation rather than development. To date, almost 200 ectodermal dysplasia syndromes have been identified, but only a limited number have been linked to mutation of a particular gene. Of those syndromes where a causative mutation has been found, mutations in both p63 as well as various desmosomal adhesion proteins have been identified,[8] suggesting the interesting possibility that Perp, as a desmosomal protein

and a target of p63, may be mutated in human ectodermal dysplasia syndromes of unknown etiology. Indeed, the characteristics of *Perp−/−* mice resemble those of human ectodermal dysplasia syndromes: keratoderma, especially in the soles of the feet, aberrant nail development, hair abnormalities, dermatitis, and a predisposition to infection, are symptoms typical of these diseases.

The symptoms observed in the *Perp−/−* adult mice were more restricted than those resulting from *p63* mutation in humans,[1] suggesting that *Perp* is only one of several p63 target genes required for the development or maintenance of ectoderm-derived tissues. Distinct mutation profiles in *p63* have been identified in different ectodermal dysplasia syndromes with overlapping but not identical symptoms, suggesting that subtle alterations in the p63 protein can affect its activity in diverse ways.[8] For example, mutations in the DNA binding domain are most commonly found in EEC syndrome, which affects many ectoderm derivatives and limb development, while mutations in the sterile alpha motif (SAM) domain are associated with AEC/Hay-Wells syndrome, in which patients generally develop many abnormalities in the epithelia but have minimally affected limb formation. A better understanding of specific genes downstream of p63 may ultimately help explain the varying effects of different mutations in this critical transcription factor, by identifying genes that are important for the development and maintenance of different ectoderm-derived tissues. Abnormalities in aging *Perp*-deficient mice are largely confined to specific stratified epithelia and suggest that Perp's function downstream of p63 is most central in the skin, hair, and nails rather than in the ectoderm-mesoderm signaling involved in limb and craniofacial development. Further investigation of genes like *Perp* will help elucidate p63 function in different spatial and temporal contexts of ectoderm specialization.

Our results offer additional insight into the transcriptional programs downstream of p53 and p63 in the differentiation and homeostasis of aging adult tissues. Loss of Perp does not increase the predisposition to spontaneous tumorigenesis, suggesting it does not participate in the suppression of tumor initiation mediated by p53. With respect to a role in the maintenance of unstressed epithelia directed by p63, Perp makes a significant contribution to the function of the skin and other appendages. These results demonstrate that in addition to an important function in adhesion within newborn epithelia, Perp is also required throughout adult life for proper epithelial homeostasis and function. Given the large number of ectodermal dysplasia syndromes and their classification by clinical symptoms, our phenotypic description of *Perp*-deficient mice may offer a good foundation for identifying corresponding human syndromes linked to disruption of Perp function.

Acknowledgements

This work was supported by the NCI (PHS Grant Number CA09302) to RAI and by the NCI (CA93665-01) and Damon Runyon Cancer Research Foundation to LDA. The authors would like to thank T Johnson, J Sage, S Artandi, P Marinkovich, and A Oro for advice and critical reading of the manuscript. We would also like to thank M Marques, S Basak, and T Johnson for mouse care and assistance with sample collection.

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