Tumor suppressors: enhancers or suppressors of regeneration?

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Summary
Tumor suppressors are so named because cancers occur in their absence, but these genes also have important functions in development, metabolism and tissue homeostasis. Here, we discuss known and potential functions of tumor suppressor genes during tissue regeneration, focusing on the evolutionarily conserved tumor suppressors pRb1, p53, Pten and Hippo. We propose that their activity is essential for tissue regeneration. This is in contrast to suggestions that tumor suppression is a trade-off for regenerative capacity. We also hypothesize that certain aspects of tumor suppressor pathways inhibit regenerative processes in mammals, and that transient targeted modification of these pathways could be fruitfully exploited to enhance processes that are important to regenerative medicine.

Key words: Regeneration, Evolution, Tumor suppressors, Differentiation, Tissue homeostasis

Introduction
Regeneration of complex tissues and organs after injury requires extensive cellular proliferation that is precisely regulated and executed in concert with differentiation and patterning mechanisms that preserve the organization of tissues within an organ. How this occurs with high fidelity, on both large and small scales, is matched in sophistication and complexity only by organ generation during development. Neoplasia represents a loss of control or absence of these processes of proliferation or differentiation, respectively. Tumor suppressor genes, initially identified because they are inactivated in mammalian tumors, were subsequently shown experimentally to protect against neoplastic transformation, and are now known to have a long evolutionary history with the ability to regulate diverse and fundamental cellular processes, including growth and division, genome maintenance, differentiation, metabolism and death (Fig. 1) (Sherr, 2004; Pearson and Sánchez Alvarado, 2008). Many tumor suppressor genes play crucial roles during normal development and postnatal life that are more likely to explain why they evolved than does their role in protection from tumors. Tumor suppressor pathways inhibit regenerative processes in mammals, and that transient targeted modification of these pathways could be fruitfully exploited to enhance processes that are important to regenerative medicine.

In this Hypothesis article, we discuss the mechanisms of action and consequences of the diverse functions of tumor suppressors during regenerative processes. In early vertebrates, the use of proliferative progenitor and stem cells for tissue homeostasis and regeneration may have also introduced a robust new source of cancers, necessitating the development tumor suppression (Pearson and Sánchez Alvarado, 2008; Belyi et al., 2010). It could be surmised that this resulted in the creation of a perpetual 'fine line' on either side of which the need for proliferation must be balanced against protection from cancer. Tumor suppressor mechanisms that police stem cells and eliminate aspiring cancer cells might be expected to impede proliferation and thus inhibit certain regenerative processes, a form of antagonistic pleiotropy (Greaves, 2007). Therein lies a potential trade-off between tumor suppression and regenerative capacity that is particularly highlighted when examining the relationships between aging, cancer susceptibility and stem cell proliferation (Kim and Sharpless, 2006; Campisi and d’Adda di Fagagna, 2007; Seifert and Voss, 2013).

Tumor suppressor genes are subject to continuous evolutionary pressure and their expression and activities are also modified in a context-dependent manner. Thus, among vertebrates, tumor suppressor pathways have variable components in different species (del Arroyo and Peters, 2005; Belyi et al., 2010) and, within a species, tumor suppressor gene function varies with developmental stage and age (Kim and Sharpless, 2006). Regenerative capacity also varies markedly among these same parameters of species, developmental stage and age (Brockes and Kumar, 2008). Although there is no readily identifiable hierarchical pattern of regenerative capacity in vertebrate evolution, lower vertebrates that are highly regenerative (such as teleost fish and urodele amphibians – salamanders) and poorly regenerative vertebrates, such as mammals, have differences in their tumor suppressor machinery that could be related to their ability to regenerate.

We propose that, in a context-specific manner, some tumor suppressor genes may exhibit 'regeneration suppressor' activities that, if understood, could be targeted to improve regenerative capacity. However, we also suggest that tumor suppressors are, in general, necessary components for regeneration that both orchestrate major aspects of the process and ensure its safety. The current emphasis in the field of regenerative medicine on cellular mechanisms of regeneration, including in vitro expansion and reprogramming, has rekindled interest in the regulation of proliferation, survival and cell death – processes that are the domain of tumor suppressor genes. This also provides an opportunity to revisit basic questions concerning how these genes function in model organisms during regeneration in vivo.

As a comprehensive review of the many tumor suppressor genes is not possible here, we focus specifically on four core tumor suppressors, retinoblastoma (pRb1), p53 (Trp53), phosphatase and tensin homolog (Pten) and Hippo (Box 1), for which substantial published data exist, and which exemplify the diverse roles of tumor suppressors during regeneration. We are primarily concerned with regeneration in vertebrates, although we also touch on data from Drosophila and planarians that illustrate relevant principles.
Susceptibility of regenerative vertebrates to tumors

It has been suggested that the evolution of more complex tumor suppressor pathways in higher vertebrates might be linked to size and lifespan: that the greater number of cell divisions in a large, long-lived animal would increase the chances of cancer. However, such arguments are not tenable. For example, although humans are large and have long lives, other mammals with similar tumor susceptibility and regenerative capacities (or deficiencies) are small and relatively short-lived. Moreover, comparison among highly and poorly regenerative vertebrates does not indicate any strong relationship between lifespan, size, regenerative capacity and cancer resistance (discussed further below). Laboratory mice live for 1-2 years in captivity, in contrast to zebrafish and axolotl, which live from 2-4 years and 10-14 years, respectively. Although zebrafish are small, axolotls are larger than mice. Therefore, when considered objectively, a general basis for a greater need for tumor suppression in higher vertebrates is not readily forthcoming. It is possible that evolutionary pressure on tumor suppressor pathways relates to environmental influences, differences in metabolism and or immune systems. Nonetheless, it is difficult to identify a well-founded teleological explanation for a trade-off relationship between cancer susceptibility and regenerative capacity, at least within vertebrates.

The notion that genetic divergence of tumor suppressor pathways among highly regenerative (e.g. teleost fish and urodeles) and poorly regenerative vertebrates (e.g. mammals) might be partly responsible for differences in regenerative capacity begs the question of whether regenerative vertebrates are susceptible to tumors. The simple answer to this is yes. However, the issue is more complex because the significant differences in environment, physiology, anatomy, metabolism, etc., mean it is of limited relevance to compare directly the incidence, penetrance and phenotype of various tumors among fish, amphibians, rodents and humans in a meaningful way. There is no question that a wide variety of tumors occur in regenerative organisms both in the wild and in captivity. Moreover, many of the tumor-promoting agents used in mammalian studies are also tumorigenic in teleosts and urodeles, and genetic models, especially in zebrafish, support the existence of conserved genetic elements related to both tumor formation and tumor suppression (Amatruda et al., 2002; Liu and Leach, 2011). Virtually all tumor types described in humans have been observed in zebrafish in captivity (Spitsbergen et al., 2012). In addition, both anuran (frogs and toads) and urodele amphibians develop neoplasms in all major organ systems (Anver, 1992). The comprehensive chapter by Green and Harshbarger in Amphibian Medicine and Captive Husbandry (Green and Harshbarger, 2001) catalogs described amphibian tumors in the wild and in captivity, bringing together multiple streams of data and applying stringent criteria, including rigorous pathological analysis, to permit confirmation of the diagnosis of ‘tumor’. Interestingly, in addition to being documented in the viscera and hematopoietic systems, spontaneously occurring benign and malignant tumors have been found in urodele tissues and in cell types that are involved in epimorphic regeneration, such as skin (papillomas and malignant melanophoromas) and mesenchymal tissue (fibromas and fibrosarcomas) (Wright and Whitaker, 2001). Taken together, the available data do not indicate a clear generalized resistance or enhanced susceptibility of urodeles or teleosts to tumor formation or to cancer compared with other animals, and show that regenerative vertebrates are susceptible to a broad spectrum of malignancies.

However, some aspects of tumor susceptibility, particularly in the contexts of exposure to chemical carcinogens and of the specific tissues undergoing regeneration and blastema formation, do suggest distinct responses at least in the urodele to factors that would induce tumor formation in mammals or adult anuran amphibians (reviewed by Tsonis, 1983; Anver, 1992; Brockes, 1998). In studies beginning in the 1940s in which chemical carcinogens were applied to salamanders, sometimes directly to the blastema (reviewed by Brockes, 1998), the surprising finding was a lower than expected incidence of malignant tumor formation, leading to the notion that urodeles have greater tumor resistance than other species. Remarkably, transplantation of established tumors into a regenerating blastema could even induce differentiation and loss of the malignant phenotype. Generally, the application of carcinogenic substances to regenerating structures in urodeles interferes with regeneration and results in sprouting of supernumerary limb buds, or other limb anomalies, but does not cause invasive tumor formation (Breedes, 1952; Tsonis and Eguchi, 1981; Tsonis, 1983; Pfeiffer et al., 1985). In the regenerating newt lens, only the regenerating dorsal iris is resistant to chemically
Box 1. Cellular functions of key tumor suppressors

p53
The p53 protein is a sequence-specific transcriptional activator that is normally expressed at low levels. p53 is stabilized and accumulates in response to various forms of cellular stress (including radiation, hypoxia, reactive oxygen species and oncogene activation), triggering downstream gene expression. Depending on the cellular context and degree of stress, activated p53 induces either growth arrest and repair, autophagy, senescence or apoptosis. The phenotypic end result is either cell recovery and survival, or death. The principal negative regulator of p53 in vertebrates is Mdm2, which can in turn be inhibited by the higher vertebrate-specific tumor suppressor Arf. Thus, Arf activates p53, causing induction of p21 (Cdkn1a) among other p53 target genes.

Retinoblastoma protein
Retinoblastoma protein (pRb1) is a ubiquitously expressed transcriptional repressor. It blocks proliferation at the G1/S transition by binding to and inactivating members of the E2F transcription factor family. Phosphorylation and inhibition of pRb1 by cyclin-dependent kinases (CDKs) releases E2F and results in entry into S phase. CDKs are in turn inhibited by the inhibitor of kinase (INK) family of proteins, of which Ink4a is a principal member and important tumor suppressor. pRb1 also controls cellular differentiation by regulating expression of tissue-specific transcription factors such as Myod1, via its association with chromatin modifiers such as histone deacetylase, PCAF (P300/CBP-associated factor) and P300.

Pten
The Pten gene product is a lipid phosphatase that dephosphorylates phosphatidylinositol (3,4,5) triphosphate (PtdIns(3,4,5)P3) at the 3 position. Pten is thus an antagonist of phosphatidylinositol-3-kinase (PI-3 kinase), the activation of which regulates Akt/protein kinase B (Akt/PKB). PI-3 kinase signaling also interacts with the p53 pathway, activating the p53 inhibitor Mdm2. Pten therefore promotes p53 activity. PTEN deficiency, and the consequent overactivation of the PI-3 kinase pathway, results in a hyperproliferative state and increased cellular survival.

Hippo signaling
The Hippo pathway is a kinase cascade whose members link cell-cell contact and cytoskeletal information to the downstream activation of gene expression that regulates cell proliferation and survival. The cytoskeletal proteins Merlin (Nf2) and Expanded indirectly stimulate the phosphorylation of the protein Warts (also known as Lats1) by Hippo (Mst1/2). Warts, in turn, interacts with phosphatases and inactivates Yap1/Tip63, a transcriptional co-activator that induces expression of genes related to proliferation and survival. Yap1 interacts with the p53 family by potentiating p73-mediated apoptosis.

Conservation of core tumor suppressor pathways
The four core tumor suppressor pathways we consider here (p53, retinoblastoma, Pten and Hippo) are evolutionarily ancient and exist in both highly and poorly regenerate organisms. By definition, tumor suppressor pathways contain one or more tumor suppressor genes, each defined by the characteristics of undergoing biallelic inactivation in tumors, conferring tumor susceptibility in individuals who inherit a single mutant allele, and being inactivated in sporadic tumors (Sherr, 2004). Discussions of each of these pathways are usually weighted towards a focus on tumor suppressor aspects, largely because mutated alleles of these genes were first identified in individuals with tumor syndromes, in tumors themselves or in mutational loss-of-function screens that yield tumor phenotypes. However, it is now understood that the core tumor suppressor pathways are also key regulators of many processes related to, but distinct from, cancer prevention. It is generally accepted that it is these other functions that are most conserved and are the targets of the selective pressures that drove their evolution.

p53
The prototypical tumor suppressor gene and pathway is p53 (Box 1, Fig. 2), and virtually all cancers either harbor mutations in the p53 gene or have functionally inactivated p53 via other key pathway components (Junktilla and Evans, 2009). Three p53 family members exist in fish, amphibians and mammals: p53, p63 (Trp63) and p73 (Trp73) (Lu and Abrams, 2006), although to date only one p53 member has been identified in urodeles (Villiard et al., 2007). Although all three can each induce growth arrest and apoptosis, p53 is the primary tumor suppressor. Unlike p63 and p73, it is frequently mutated in human and mouse cancers, and results in a tumor prone phenotype when depleted from or disrupted in the germline (Malkin et al., 1990; Donehower et al., 1992). Surprisingly, p53 is the newest family member: the ancestral member in single cell choanoflagellates and metazoan sea anemones is more closely related to p63 and p73 (Belyi et al., 2010). No p53 family members have been identified in prokaryotes or yeast. It has been proposed that the evolutionary appearance of p53 as distinct from the primitive p63/p73-like ancestor coincides with the emergence of adult somatic tissue stem cells in which it functions as a tumor suppressor by policing DNA damage (Belyi et al., 2010), whereas the ancestral gene in hydra, nematodes and
flies functions to maintain genomic fidelity in the germline (Belyi et al., 2010; Dötsch et al., 2010). Notably, p53-null flies do not develop tumors (Kondo, 1998; Sogame et al., 2003). In planaria, a highly regenerative multicellular organism, one p53 ortholog has been identified and shown to have important functions in stem cell self-renewal and differentiation, as in vertebrates (Pearson and Sánchez Alvarado, 2010). In vertebrates, p63 and p73 perform distinct functions in embryogenesis of the skin and appendages, and the immune and nervous systems, respectively. In addition, they retain the ancestral role of ensuring germ cell fidelity (Suh et al., 2008). The ancient germline protective function of p53 orthologs is conserved, and the newer tumor suppressor function that is the principal function of p53 exists in multicellular creatures that have adult tissue stem cells, including planaria, and both highly and poorly regenerative vertebrates (Villiard et al., 2007) (Lu and Abrams, 2006; Belyi et al., 2010).

**Retinoblastoma proteins**

The retinoblastoma proteins pRb1 (p105), p107 and p130 are central to regulation of the G1/S transition of the cell cycle (Box 1, Fig. 2), and are at the focal point of the central growth control retinoblastoma pathway. Like p53, Rb1 (the gene encoding pRb1) has two other family members in mammalian cells, p107 (Rbl1) and p130 (Rbl2) (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993). The retinoblastoma pathway is also largely conserved in eukaryote evolution and homologs exist in plants and animals. However, the ancestral member of this family appears to be Rb1, because, at the amino acid level, nematode and Drosophila homologs most closely resemble pRb1. No retinoblastoma protein relatives have been identified in unicellular organisms. Human Rb1 was first identified as a tumor suppressor mutated in children who develop retinal tumors (Knudson, 1971; Cavenee et al., 1983; Friend et al., 1986). However, it was subsequently found to have broad functions in promoting tissue differentiation and maturation during embryogenesis. Deletion of Rb1 in the mouse germline results in an embryonic lethal phenotype that is characterized by defects in development of the CNS, hematopoiesis and skeletal muscle (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Rb1 deficiency results in inappropriate cycling of cells and failure of terminal differentiation and expression of mature tissue specific gene expression profiles. The ‘pocket domain’ (that mediates binding to other proteins) of pRb1 is highly conserved in vertebrates (Lee et al., 1998). Its biochemical functions are also conserved in zebrafish and urodeles, although the role of pRb1 as a tumor suppressor is not well documented.
suppressor has not been studied in these species (Tanaka et al., 1997; Thitoff et al., 2003; Rios et al., 2011; Gyda et al., 2012). Although inactivation of the retinoblastoma pathway is a prerequisite for tumor formation, the crucial role of Rb1 in forming normal tissues suggests that evolutionary selection has probably operated primarily on its roles during embryogenesis, rather than on its postnatal tumor suppressor functions. Conversely, a key modulator of pRb1, the cyclin-dependent kinase inhibitor Ink4a (Cdkn2a), is a mammalian tumor suppressor that is frequently mutated in cancers, but has no crucial role in development, as evidenced by the viability, fertility and lack of structural defects in most tissues of Ink4a-null mice (Serrano et al., 1996).

**Pten**

Pten is another tumor suppressor gene very commonly mutated in human cancers (Li et al., 1997; Steck et al., 1997). A negative regulator of PI-3 kinase signaling, Pten constrains cell size, number and survival (Box 1, Fig. 2). Pten is essential during development and Pten deficiency results in embryonic lethality at day 8.5 in mice (Di Cristofano et al., 1998; Sun et al., 1999). Heterozygous mice or mice conditionally mutant for Pten develop hyperplasia, and exhibit reduced apoptosis and overgrowth phenotypes in multiple tissues and organs. Similar phenotypes are also observed upon mutation of the Drosophila, nematode, planaria, Xenopus and zebrafish Pten homologs (reviewed by Cid et al., 2008). Pten has not been identified and functions have not yet been studied in urodele amphibians. The zebrafish and planaria genomes each contain two Pten genes: in planaria, these appear to be fully redundant, whereas the zebrafish paralogs have undergone some functional divergence (Croushore et al., 2005; Faucher et al., 2008; Oviedo et al., 2008).

**Hippo signaling**

Among the newest major growth control pathways to be identified is the Hippo signaling pathway (Box 1, Fig. 2), with the major negative modulator of this pathway, the Hippo gene, first discovered in 2003 in Drosophila (Wu et al., 2003) – the species in which this pathway has been best characterized. Although Hippo was discovered in ‘tumor suppressor’ screens in Drosophila, the selected phenotypes are for overgrowths and tumor-like formations, not for invasive cancer. However, inactivating mutations in the upstream regulator Merlin (neurofibromatosis 2; N2) lead to Schwann cell tumors and meningiomas in humans. And the downstream effector of the pathway, Yap1 (yes-associated protein 1) is an oncogene that is frequently overexpressed in a wide spectrum of human cancers and that induces hepatocellular carcinomas when overexpressed in mice (reviewed by Pan, 2010). Available evidence indicates that the tumor suppressor activity of the pathway is perhaps less pervasive than that of p53, pRb1 and Pten, and that the function of Hippo in organ size control and other aspects of development predominate. The fundamental roles of Hippo signaling in Drosophila development, first recognized in the restriction of growth by Hippo of the imaginal discs (the epithelial primordia of adult structures), are broad and affect many tissues and organ systems, including the ovarian follicle and the CNS. The essential cellular functions of Hippo signaling include coordination of the transition from proliferative to postmitotic states and full expression of the differentiated phenotype (reviewed by Pan, 2010). In zebrafish and Xenopus, Yap1 is expressed ubiquitously during embryogenesis (Jiang et al., 2009; Nejigane et al., 2011), and in zebrafish it is required for development of the brain and craniofacial structures (Jiang et al., 2009).

**Recent innovations in tumor suppressor pathways might alter regenerative capacity**

As discussed above, the core tumor suppressor pathways are highly conserved throughout evolution and are present in both highly and poorly regenerative species. Although genomic information is limited for urodeles, retinoblastoma proteins and p53 have been identified, and their protein products have been confirmed to function in at least some respects like their counterparts in other species. Moreover, available information confirms similar, if not completely conserved, core functions for the p53, retinoblastoma, Pten and Hippo pathways in the other highly regenerative species, including planaria and the vertebrate zebrafish. However, there are potentially important differences in the pathways between highly regenerative species and poorly regenerative higher vertebrates, such as the diversification of the retinoblastoma and p53 pathways. Moreover, when more sophisticated modulators of the pathways are considered, key differences could underlie important divergences among highly and poorly regenerative species. One intriguing example is the evolutionary appearance of the Arf (Cdkn2a) tumor suppressor (see Box 1 and Fig. 3) in non-regenerative vertebrates. As discussed above, the mammalian Ink4a locus is a major tumor suppressor. In mammals, the Ink4a locus has a highly unusual if not unique organization that results in the production of two proteins, Ink4a and Arf, via separate first exons and common second and third exons (Quelle et al., 1995). The two proteins are encoded by alternative reading frames and have no amino acid homology and have completely distinct biochemical functions, but both are potent tumor suppressors in mice and humans. Whereas homologs of Ink4a (a component of the retinoblastoma pathway) exist in fish, amphibians and lower metazoans, the earliest Arf (a component of the p53 pathway) ancestor arose in birds (Gilley and Fried, 2001; Kim et al., 2003; del Arroyo and Peters, 2005). Potential implications relevant to regeneration have been proposed and are discussed below.

As another example, the p53 target p21 (Cdkn1a) acts as a negative regulator of cyclin-dependent kinase activity. The p21 gene does not appear to exist in planaria (Pearson and Sánchez Alvarado, 2010) but is present in zebrafish (Langheinrich et al., 2002). p21 has been implicated in suppression of epimorphic regenerative capacity in a mouse ear hole model (Bedelbaeva et al., 2010), whereas deficiency of p53 had no apparent effect (Arthur et al., 2010). Although the existence of p21 in zebrafish demonstrates that potent regeneration can occur in organisms that contain this gene, it is possible that differences in regulation of p21 expression, e.g. the presence or absence of Arf (see Box 1), or in Tgfβ/Smad activity (Pardali et al., 2000) could limit expression of p21 during regeneration in zebrafish. This possibility has yet to be tested experimentally. Undoubtedly, other evolutionarily divergent genes will be identified that modulate the core pathways in important ways, and presumably have evolved to perform functions uniquely important in the species in which they exist. Whether they also result in physiological side effects that alter other functions such as regeneration remains to be tested.

**Core tumor suppressor pathways are required for tissue regeneration**

Unlike cellular proliferation that replenishes the hematopoietic system or re-epithelializes skin wounds, regeneration of other complex tissues and organs after injury involves coordinated
developmental functions during regeneration. However, it is also clear that regeneration involves certain processes that are distinct from embryonic development, such as the initial wound healing step, and activation of quiescent progenitors or reassignment of terminally differentiated cells to execute the regenerative process. Tumor suppressors could have regeneration-specific functions in these processes.

General physiological processes are frequently recapitulated in regeneration, and the specific activities of tumor suppressors may be similarly conserved. One example of this phenomenon is provided by the role of p53 in the injury response in Drosophila Imaginal discs. Imaginal discs undergo regeneration after injury, in a process involving compensatory proliferation that is stimulated by injured, dying or dead cells (reviewed by Worley et al., 2012). The proliferative response is the end result of a multistep process that first requires cell cycle arrest, autocrine and paracrine signaling [including induction of the Wingless (Wg) pathway]. Interference with any of these steps has been shown to block compensatory proliferation, and dp53 function is crucial for regeneration, as dp53 mutants fail to induce G2 arrest, wg expression or compensatory proliferation (Wells et al., 2006). Thus, in this context, p53 appears to promote rather than restrain proliferation. The authors of that study suggest that these dp53 functions, which appear to be at odds with tumor suppressor functions of mammalian p53, may relate more closely to functions that have been adopted by p63 during vertebrate epidermal development (Wells et al., 2006). The important role of p63 in promoting epidermal progenitor cell renewal (Koster et al., 2004) suggests that parallels exist between mechanisms of p53 family functions in regeneration and uninjured tissue formation and renewal.

That tumor suppressors would be involved in regulating the proliferation-dependent aspects of regeneration is to be expected given their known molecular functions, although the example above suggests that even in this context, their roles may be more complex than might be anticipated. However, there is also evidence for roles of tumor suppressors in regulating regeneration independently of proliferation, notably in the case of peripheral nerve axon regeneration. Given the nerve dependence of regeneration of limbs and other tissues (Singer, 1974), this is particularly important to the biology of regeneration of complex tissues. p53 was found to be necessary for central and peripheral nerve regeneration, a function not necessarily predicted from developmental studies of p53 (Di Giovanni et al., 2006). As axon and dendrite regeneration occur in the absence of neuronal cell division, the dependence on p53 for neurite outgrowth in culture and peripheral nerve regeneration in vivo in mice represents an unexpected activity of this tumor suppressor. p53 targets activated in this context, coronin 1b (Coro1b) and Rab13, are distinct from those involved in growth arrest or apoptosis. Although CNS defects frequently occur in p53 knockout mice (Armstrong et al., 1995), it is not yet clear whether these developmental phenotypes are a result of the pro-apoptotic function of p53, or whether they reflect an activity conserved between the developmental and regeneration contexts.

The only highly regenerative multicellular organism in which the role of Pten has been examined in the context of tissue regeneration is planaria. The mechanisms of regeneration in planaria differ significantly from those in vertebrates, primarily owing to the existence of a pluripotent cell population, the neoblasts, dispersed throughout the body of the adult flatworm (Newmark and Sánchez Alvarado, 2000; Newmark and Sánchez Alvarado, 2002; Reddien and Sánchez Alvarado, 2004) – no equivalent of which exists in vertebrates. However, as in the
highly regenerative teleosts and urodèles, planaria regeneration involves the formation of a blastema (an epithelium-covered mass of proliferating mesenchymal cells that will undergo morphogenesis and patterning during regeneration) and although these are not fully equivalent structures, the molecular and cellular processes controlling proliferation and differentiation may share significant similarities with those occurring during vertebrate regeneration. Amputation experiments in planaria in which the two Smad-Pten genes were suppressed using RNAi resulted in inappropriate proliferation of neoblasts, failure of both differentiation and tissue regeneration, and the production of abnormal tissue outgrowths (Oviedo et al., 2008). This striking example underscores the importance of limiting proliferative responses during regeneration and demonstrates a necessary function of Smad-Pten in forming normal tissues during regeneration. Although the role of Pten has not yet been examined in vertebrate epimorphic regeneration, a very different role for this tumor suppressor has been identified in mammalian peripheral and central nerve regeneration (Park et al., 2008; Liu et al., 2010; Sun et al., 2011) (discussed below).

As in the case of Pten, one would predict that loss-of-function experiments targeting Rb1 or Hippo would also impair regeneration of complex tissues by disrupting patterning or differentiation – as both factors regulate these processes during development. There is evidence from Drosophila and mouse studies that the Hippo pathway is a regulator of intestinal, epidermal and neural progenitor function. Moreover, after intestinal or liver injury, components of the Hippo pathway are important regulators of repair (reviewed by Zhao et al., 2011). The crucial role of Rb1 in the transition from a proliferative state to a postmitotic differentiated one has been amply demonstrated in developmental studies in mice (Burkhart and Sage, 2008) and in studies of muscle cells in culture (Gu et al., 1993; Lassar and Münsterberg, 1994; Novitch et al., 1996). The failure of organized hematopoiesis, neurogenesis and myogenesis in the absence of Rb1 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992) strongly suggests that similar outcomes would be observed if Rb1 were rendered deficient during tissue regeneration. Moreover, cell culture experiments in which suppression of Rb1 was used to induce muscle cell proliferation required re-establishment of Rb1 function to induce differentiation of myoblasts and incorporation into muscle (Pajcini et al., 2010).

Considering the significant use of developmental programs during tissue regeneration, together with the experimental regeneration data available to date, leads us to conclude that tumor suppressor function is a likely requirement for regeneration of complex tissues. Some of these requirements relate to the known functions of tumor suppressors in orchestrating proliferation, differentiation and cell death, whereas newly recognized functions, such as the promotion of axon regeneration by p53, are also being revealed.

Putative regeneration-promoting functions of tumor suppressors
Considering the known tumor suppressor activities of p53, pRb1, Pten and Hippo generates some hypotheses for how these genes and pathways might be predicted to function to support regeneration (Fig. 1).

Tumor suppression
First, it is easy to speculate that tumor suppressors would suppress tumorigenesis during the periods of extensive and rapid cellular proliferation that occur during regeneration. For pathways such as Hippo and Pten, one could predict that the known function of organ size regulation would be harnessed to restrain and organize tissue growth during regenerative re-growth – preventing tumor formation. For p53 and pRb1, the classical tumor suppressor functions of preventing inappropriate proliferation and eliminating or enabling repair of stressed or genome-damaged cells would be expected to be important for ensuring a healthy and tumor-free regenerate. In mammals, the occurrence of carcinomas in chronically regenerating liver and skin presumably represent rare failures of these functions in the setting of the long-term presence of proliferative signals. Although the absolute numbers of cell divisions involved in newt limb regeneration do not approach those seen during normal turnover of mammalian skin occurring over a lifetime, it is still remarkable that a newt can successfully regenerate amputated limbs many times, each involving massive proliferation over a relatively short period. That newt limb regeneration proceeds without tumor formation, implies that potent tumor suppressive functions must be involved during the regenerative process.

Promotion of differentiation
The tumor suppressor genes discussed in this Hypothesis have all been shown to promote differentiation – a potent anti-cancer activity – in various contexts, and this would have an obvious role during regeneration. Just as crucial for regeneration as the formation of a highly proliferative blastema is the process of inducing cell cycle exit and the expression of genes that characterize the mature differentiated phenotype. As mentioned above, Rb1 is the classic example of a gene that performs this function (Pajcini et al., 2010). Experiments that disrupt retinoblastoma protein function in a regenerative setting during or after the blastema stage have not yet been performed but would be important to demonstrate the importance of this function and its conservation from embryogenesis to regeneration.

Induction of apoptosis
Induction of apoptosis is a central function of p53 in tumor suppression (Voussen and Prives, 2009) and can also be attributed to other tumor suppressors in certain contexts (Basu et al., 2003; Lehman et al., 2011). Apoptotic activity is particularly prominent during limb formation, to separate the digits by eliminating the interposed epidermal and mesenchymal tissue (Zuzarte-Luis and Hurle, 2005). This raises the issue of whether the classical tumor suppressor pathways are involved in this process. There is no evidence to date of a role for tumor suppressor pathways in regulating interdigit programmed cell death, either in development or regeneration, but the apoptotic machinery downstream of p53 family members [Bax, Bak1, Bid, Bim (Bcl2I11) and Puma (Bbc3)] is known to control this process (Lindsten et al., 2000; Ren et al., 2010). Although it has long been known that interdigit programmed cell death is mediated by BMP signaling (Hernández-Martinez and Covarrubias, 2011), the immediate upstream communication to the Bcl2 family in such cases is unclear. Normal limbs and digits form in p53-deficient mice, but p63 may be a better candidate for regulating apoptosis in this context, as it has important epithelial and appendage developmental roles and can activate Bax-dependent apoptosis in other contexts (Gressner et al., 2005). Moreover, Pten and Hippo can cooperate with p53 family members (p73 in this case) to regulate apoptosis (Basu et al., 2003; Lehman et al., 2011). Limb development and regeneration therefore provides an ideal setting.

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in which to analyze the potential pro-apoptotic functions of tumor suppressors during regeneration.

**Metabolic regulation**

Finally, another potentially important possible function of p53 during regeneration relates to its more recently recognized roles in metabolism – primarily discussed in the context of tumorigenesis (reviewed by Gottlieb and Vousden, 2010). Under conditions of daily life and during stress, rather than eliminate cells, p53 functions to lower reactive oxygen species (ROS) levels and to promote DNA repair and cellular survival. After injury, oxygen, lactate and ROS levels are drivers of healing and regeneration (Trabold et al., 2003; Thom, 2009), and it has been shown that tight regulation of elevated ROS is crucial for tail regeneration in *Xenopus* (Love et al., 2013). As such, the regeneration milieu may be considered a ‘low stress’ environment in which p53 could function to promote survival (rather than trigger apoptosis) (Fig. 1). Whether cells in the regenerate undergo a type of metabolic transformation similar to that seen in cancer (Gottlieb and Vousden, 2010) remains to be seen. Although experimental evidence is currently lacking, it is tempting to speculate that p53 could promote cellular survival and maintenance during regeneration as it can in cancer (Kim et al., 2009).

**Putative ‘regeneration suppressor’ roles for tumor suppressors and potential exploitation in regenerative medicine**

Although solid tissue regeneration requires the restoration of architecture and the interaction between different cell types, *in vitro* studies have primarily focused on cellular regeneration or reprogramming coupled to proliferation to produce copies of a single cell type. Conceptually translating *in vitro* properties to reflect *in vivo* functions carries significant caveats, but *in vitro* data have formed the basis of many tumor suppressor studies, corroborating and, in some cases, presaging *in vivo* findings. Distinct growth properties *in vitro* have also been identified in cells from highly regenerative vertebrates that could signify important differences from mammalian cells that may impact regeneration *in vivo*. For example, urodele blastema cells can replicate indefinitely without senescence in culture (Ferretti and Brockes, 1988), and cellular senescence in the regenerative zebrafish is much slower than in mammals (Kishi et al., 2003). As senescence signals in mammalian cells are transmitted and executed through the retinoblastoma and p53 pathways (Ben-Porath and Weinberg, 2005), differences in retinoblastoma protein and p53 regulation could be expected to influence distinct senescence phenotypes of highly versus poorly regenerative species, an issue that could easily be addressed in cell culture experiments.

Understanding how cell growth and division are controlled in cultured cells may inform regeneration studies in two broad ways. First and most direct is the potential to use this knowledge to improve cell-based approaches in regenerative medicine, where the emphasis lies in the ability to produce desired cell types in abundance. Although the importance of tumor suppressor genes in stem cells, especially in relation to aging, has been well studied and reviewed elsewhere (Sharpless and DePinho, 2007), other examples are illustrative here. For example, the derivation of induced pluripotent stem cells (iPSCs) from differentiated somatic cells, a process that requires extensive proliferation and extensive reprogramming of gene expression, must overcome the significant roadblock of *in vitro* senescence. Senescence in this context, as in others, is induced by the core tumor suppressor pathways, and, conversely, suppression of tumor suppressor function can significantly increase the yield of iPSC colonies – as has been shown for Ink4a/Arf and p53. Although iPSC colonies proliferate indefinitely once they are formed, bypassing or overcoming Ink4a/Arf and p53 function appears to be a crucial step to gain proliferative capacity (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). Another approach under investigation is the expansion of committed cell populations by transient interference with tumor suppressor function, thereby conferring growth capacity while retaining the original identity (Blau and Pomerantz, 2011). By contrast, a potential hazard of *in vitro* reprogramming and culture expansion approaches is genomic instability and potential induction of tumorigenicity, and increased dosage of tumor suppressor genes may reduce this danger (Menendez et al., 2012).

Second, comparing differences in evolved tumor suppressor genes and pathways among species may shed light on differences in intrinsic *in vivo* regenerative capacities. The study of the process of cellular dedifferentiation in newts identified the retinoblastoma pathway as a key regulator (Tanaka et al., 1997), and this mechanistic understanding, coupled with the absence of the Arf tumor suppressor in highly regenerative species, led to experiments resulting in the ability to mimic newt cellular behaviors in mammalian muscle cells (Pajcini et al., 2010). Whether the absence of ARF in highly regenerative vertebrates contributes to their increased potential *in vivo* is currently being examined.

Finally, breakthroughs with direct clinical implications have been made in targeting tumor suppressors *in vivo* to enhance axonal regeneration by mechanisms distinct from effects on proliferation. A number of studies have demonstrated that axon regeneration of central and peripheral neurons can be significantly enhanced by interfering with the expression of Pten after crush injuries (Park et al., 2008; Liu et al., 2010; Sun et al., 2011). Interestingly, targeting of p53 in similar experiments promoted neuronal survival but did not enhance regeneration (Park et al., 2008). These remarkable findings in mice established an important link between control of protein synthesis by the Pten pathway and regeneration, a link not easily predictable in the context of canonical PTEN tumor suppressor functions.

**Conclusion**

The diverse functions of the tumor suppressors discussed in this Hypothesis article defy the simple model that tumor suppressors restrict proliferation, are therefore anti-regeneration, and can be bluntly targeted to enhance regeneration. Instead, from the available regeneration data reviewed here along with known functions in relevant contexts such as development and postnatal metabolic regulation, we conclude that tumor suppressors in general support regenerative processes. This view holds both in terms of tumor suppressor roles in modulating proliferation and differentiation, as well as in protecting cellular genomic integrity that is likely at risk during periods of metabolic and proliferative stress. In fact, enhancing tumor suppressor function in the right temporal context may be important in future efforts to induce regenerative processes. However, it is also clear in the case of Arf (which does not exist in highly regenerative vertebrates such as fish and amphibian) that some key tumor suppressor genes are not required for efficient regeneration without malignant transformation. Moreover, the existence of multiple family members, as with p53 and retinoblastoma genes, may allow certain family members to have taken on specific roles during regeneration. These observations could indicate opportunities to
selectively modify tumor suppressor activities to improve desired regenerative outcomes. Indeed, the experiments described in this article support this view and have already shown that inhibition of tumor suppressor function can productively permit dedifferentiation, reprogramming and expansion of desired cell types.

It remains to be seen whether perturbation of tumor suppressor function can enhance regeneration of complex tissues in vivo, as has been shown for regeneration of somatic stem cells in the hematopoietic system, endocrine cells of aged animals (Janzen et al., 2006; Krishnamurthy et al., 2006) and axons (Park et al., 2008; Liu et al., 2010; Sun et al., 2011). Additional loss-of-function experiments in mammals may resolve this. In zebrasfish and salamanders, the introduction of mammalian tumor suppressors such as Arf during regeneration will determine whether they harbor true regeneration suppressor activity. In the context of in vitro cellular regeneration protocols, the transient targeting of select tumor suppressors during windows of opportunity may eventually elicit expansion without negative consequences. A sophisticated understanding of the complex functions of tumor suppressors in distinct contexts is merited and will most certainly continue to be informative for regenerative medicine applications and basic questions alike.

Acknowledgements
The authors apologize to colleagues whose important work was not cited owing to space limitations. We would like to thank Robert Hesse and other members of our laboratories for insightful comments on the manuscript.

Funding
J.H.P. is supported in part by funds from the University of California San Francisco Departments of Surgery and Oroficial Sciences; the University of California San Francisco Program for Breakthrough Biomedical Research Opportunity Award; and a California Institute of Regenerative Medicine (CIRM) New Faculty Physician Scientist Award. H.M.B. is supported by National Institutes of Health (NIH) grants; a subcontract from the University of

Competing interests statement
J.H.P. and H.M.B are scientific founders of Didimi, Inc.

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