

Review

The role of MDM2 and MDM4 in breast cancer development and prevention

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The major cause of death from breast cancer is not the primary tumour, but relapsing, drug-resistant, metastatic disease. Identifying factors that contribute to aggressive cancer offers important leads for therapy. Inherent defence against carcinogens depends on the individual molecular make-up of each person. Important molecular determinants of these responses are under the control of the mouse double minute (MDM) family: comprised of the proteins MDM2 and MDM4. In normal, healthy adult cells, the MDM family functions to critically regulate measured, cellular responses to stress and subsequent recovery. Proper function of the MDM family is vital for normal breast development, but also for preserving genomic fidelity. The MDM family members are best characterized for their negative regulation of the major tumour suppressor p53 to modulate stress responses. Their impact on other cellular regulators is emerging. Inappropriately elevated protein levels of the MDM family are highly associated with an increased risk of cancer incidence. Exploration of the MDM family members as cancer therapeutic targets is relevant for designing tailored anti-cancer treatments, but successful approaches must strategically consider the impact on both the target cancer and adjacent healthy cells and tissues. This review focuses on recent findings pertaining to the role of the MDM family in normal and malignant breast cells.

Keywords: MDM2, MDM4, TP53, p53, breast cancer

Introduction

Cancer of the breast is among the leading causes of cancer-deaths in women in developed countries. Understanding susceptibility offers scope for prevention and effective intervention. Defining the molecular peculiarities of breast development that contribute to this risk has been revolutionized with the advent of molecular technologies.

The mouse double minute (MDM) family members have been identified as fundamental regulators of developing tissue fate and cancer protective responses. Their negative control of the

major tumour suppressor p53 highlights their risk for cancer development, including breast malignancies. Indeed, elevated levels of the MDM family members MDM2 and MDM4 are associated with breast cancer (BrC). In this article, we review the contemporary understanding of the vital normal functions of the MDM family and discuss new evidence for their deregulation. We survey consequent ramifications of their abnormal levels in BrC and summarize targeted therapies under development.

Proper MDM family function is necessary for healthy breasts

The MDM family is comprised of the E3 ligase MDM2 and its close homologue MDM4 (alternatively termed MDMX). The nomenclature 'MDM' has also been adopted for the human equivalent in the HUGO system, rather than discriminating 'HDM'. The MDM family is located in the epithelial cells, lining the lumen of the breast milk ducts in the adult breast, but are not evident in the surrounding stroma (Haupt et al., 2015).

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MDM2 is a vital regulator of tumour suppressor p53 activity in the breast

The MDM family is best characterized for its dynamic negative regulation of the major tumour suppressor p53 (Haupt et al., 1997; Kubbutat et al., 1997). Key to this regulation is the E3 ligase activity of MDM2 (Honda et al., 1997), which is moulded by MDM4. Together they act to ensure timely and measured stress responses (Karni-Schmidt et al., 2016).

In healthy cells, including mature breast epithelial cells, genomic integrity is maintained through the integrated response of the MDM family and the major tumour suppressor p53. In reaction to DNA damage (as provoked by exposure to carcinogens or radiation), molecular modifications (e.g. phosphorylation) are primed to relieve p53 from the inhibitory effects of MDM2 (Manfredi, 2010). MDM2 is consequently ubiquitinated and destroyed through the proteasome, which allows simultaneous p53 accumulation and activation of remedial pathways.

Activated p53 triggers growth inhibition and also potentiates networks of pathways to mend DNA damage in instances where repair is possible (Biegging et al., 2014). The best-characterized role for p53 is to serve as a transcription factor that can activate the expression of many target genes, including *MDM2* and *MDM4* (more specifically the full-length form, MDM4-FL, as discussed below). In response to p53 activation, levels of the MDM family subsequently increase again, reinforcing an oscillatory rhythm to the feedback loop function and allowing for a return of p53 to basal levels in the absence of stress. In the absence of stress, in healthy adult tissues, low p53 levels are maintained by MDM2 in conjunction with MDM4. They act to continuously tag emerging p53 protein with ubiquitin, which in turn destines it for proteasomal degradation [(Phillips et al., 2010) and references within it]. Consistently, in normal, healthy adult breast ducts, the major tumour suppressor p53 was not detectable, while MDM2 and MDM4 were at high levels (Haupt et al., 2015).

In addition to the vital regulation of p53 by MDM2 in normal breast, additional anti-tumourigenic functions are now emerging. MDM2 also mediates the degradation of microtubule-associated protein (haematopoietic PBX-interaction protein, HPIP). HPIP is a positive regulator of oestrogen-mediated AKT signalling, which is frequently deregulated in cancer. The risk for cancer posed by deregulation of this pathway emphasizes the importance of proper MDM2 regulation of HPIP and in turn the AKT pathway (Shostak et al., 2014).

In the normal breast epithelia, an additional level of complexity to the regulatory functions of the MDM family is added by the existence of functionally diverse isoforms of its members. Specifically, in breast epithelial cells, MDM2 exists in two main isoforms: MDM2 full-length (MDM2-FL) is ~90 kDa molecular weight and a shorter version MDM2-B is ~50 kDa molecular weight (Gudas et al., 1995).

MDM2-FL acts as the key E3 ligase and negative regulator of p53. MDM2 maintains p53 at almost undetectable levels in normal, unstressed cells. Transcription of MDM2-FL can be driven from two distinct promoters. Constitutive levels of MDM2 are maintained through transcription driven from the upstream promoter (P1), while a second promoter (P2 located in intron 1) is

commandeered in response to stimulation by p53 and also mitogens, resulting in inducible transcription (reported to increase with ~8-fold greater generation efficiency from P2 of MDM2-FL) (Phillips et al., 2010).

In response to stress signals (including genotoxic stress), alternative splice forms are also provoked and their interaction with MDM2-FL (but not p53 directly) can foster p53 stabilization (Jacob et al., 2014). Specifically, when MDM2-FL is engaged by MDM2-B at sub-equivalent stoichiometric levels, they sequester into the cytoplasm, away from the nucleus, which in turn allows p53 to accumulate both in humans (Evans et al., 2001) and mice (Dang et al., 2002). Disruption of the proper function of the MDM family can lead to a failure to properly repair or eliminate damaged DNA, which could in turn initiate BrC onset.

MDM4 regulates cellular stress responses through both MDM2-dependent and independent functions

MDM4 contributes to p53 inhibition by suppressing its transcriptional activity, and also by partnering with MDM2 to regulate p53 degradation (Francoz et al., 2006). MDM4 lacks an E3 ligase activity, but through heterodimerization it regulates MDM2 enzymatic proficiency. MDM4 does not homodimerize with high affinity, while MDM2 homodimers do form, but with less affinity than MDM4–MDM2 heterodimers. A current model indicates that the efficiency with which MDM2 ubiquitinates p53 is enhanced when it heterodimerizes with MDM4. In normal breast epithelial cells, MDM4 levels range between 10% and 20% of MDM2 levels. Imbalance in these levels defines a risk for BrC, as discussed below (Wade et al., 2013).

Wild-type (wt) p53 activation in response to stresses not only results from its release from the MDM2 but is compounded by MDM2-mediated degradation of MDM4. In turn, like MDM2, MDM4 has also been identified as a transcriptional target of wt p53 in response to DNA damage. When prompted by genotoxic and oncogenic stresses, wt p53 can bind to the MDM4 promoter region P2, in the first intron, and promote transcription. The weak promotion of this promoter by p53 suggests that additional transcription factors are likely to be involved, which in turn may impart discrimination between cells lacking or containing these factors. However, efficiency of translation of the mRNA from this promoter is greater than that from P1.

Alternative spliced variants of MDM4 also exist in the breast. MDM4-ALT2 is able to dimerize with either MDM4-FL or MDM2-FL. As with MDM2-ALT1, MDM4-ALT2 expression in response to stress promotes activation of wt p53 (Jacob et al., 2014). Deregulated generation of these splice variants poses risks for cancer (Lenos et al., 2012), as discussed below.

Stress and the hormone oestrogen receptor- α regulate MDM2 in the normal breast

The efficacy of the oscillatory cycle between MDM2 and p53 has been linked to MDM2 SNP309 that is encoded in its promoter (Bond et al., 2004). Importantly, it is located at the site of engagement of the SP1 transcription factor (a hormone coactivator) and mediates its oestrogen receptor (ER) response (Hu et al.,

2007): where oestrogen can boost MDM2 levels [(Brekman et al., 2011) and references within it].

The nucleotide composition of SNP309 impacts on MDM2 levels. SNP309 with at least a single T nucleotide promotes an efficient oscillatory response, allowing stress stimulation of MDM2, then a return to basal levels. In contrast, SNP309 G/G corresponds to higher binding affinity for the Sp1 transcriptional activator, elevated MDM2 expression levels, and in turn reduced efficiency of the p53 stress response (Hu et al., 2007). Intriguingly, this correlation is further refined in the simultaneous presence of an additional SNP285C, which reduces Sp1 affinity (Knappskog et al., 2011). Due to their impact on the potency of MDM2 function and in turn p53 activity, these SNPs have ramifications for cancer susceptibility (as discussed below). Adding another dimension, Mdm2 regulates ER α levels. MDM2 can promote transcription of the ER α gene leading to increased levels of ER α , in a p53-independent manner (Kim et al., 2011; Brekman et al., 2011; Swetzig et al., 2016). Conversely, MDM2 appears to drive the proteasomal degradation of both p53 and ER α when they co-complex, while in contrast, both become stabilized in response to stress (Duong et al., 2007).

MDM2 dictates normal breast morphogenesis and architectural sculpting of the ducts

The breast is one of the most developmentally dynamic tissues in the body. As uncontrolled proliferation is a characteristic of cancer, rigorous monitoring of cell proliferation during breast morphogenesis must be crucial. At a whole body level, the absolute interdependence between the MDM family members and p53 in viable tissue development is unequivocally demonstrated by the ability of p53 knock-out to rescue the embryonic lethality that results from the loss of either MDM2 (Jones et al., 1995; Montes de Oca Luna et al., 1995) or MDM4 (Parant et al., 2001), as evidenced in mice with independent genetic ablation of each family member. The salient features of these partnerships are that p53 is a potent inducer of cell growth inhibition and cell death and that the MDM proteins keep these activities leashed.

The MDM family and p53 are also vital dictators of normal breast morphogenesis. Coinciding with puberty, in normal developing breasts, hormone stimulation (particularly oestrogen) promotes duct proliferation, and these become more highly branched with maturity, which is regulated by progesterone. The importance of strict regulation of MDM2 in the developing breast has been demonstrated in a transgenic mouse mammary fat pad model (Lundgren et al., 1997).

Forced MDM2 overexpression in mammary epithelia led to underdevelopment and interrupted morphogenesis of the mammary gland, in a transgenic model under the bovine β -lactoglobulin promoter. Overexpressed MDM2 during lactation resulted in fewer epithelial cells and fewer alveoli. This was coincident with increased polyploidy of the epithelial cells, resulting from multiple circuits through S phase without synchronized cell division: indicative of S phase uncoupling from mitosis. This phenomenon was not countered by a wt p53 background or impacted by p53 deletion. This study exposed that in addition to vital p53 dependency,

MDM2 also has critical independent functions for normal breast development.

The impact of high MDM2 levels on lactation was so detrimental that pup-fostering was necessary. The mechanism underlying the impact of MDM2 overexpression on the mammary gland during lactation is not completely understood. It has been shown to be p53 and E2F1-independent, but dependent on its interacting partner p14ARF (p19Arf in humans) (Lundgren et al., 1997). This effect of MDM2 on lactation was enhanced by p19Arf loss, which sequesters MDM2 into the nucleolus under stress conditions (Maggi et al., 2014). The speculated explanation is that in the absence of Arf interaction, MDM2 is more freely available to interact with alternative protein partners (Foster and Lozano, 2002), including MDM4 (Riley and Lozano, 2012). While weaning efficacy and clearing through involution appeared largely unaffected by MDM2 overexpression, it is likely that residual, persistent polyploidy cells are the origin of increased adenocarcinoma risk (Lundgren et al., 1997).

While these p53-independent activities of MDM2 are vital for proper breast development (Lundgren et al., 1997), a significant role for p53 has also been shown in sculpting the breast epithelium. In the virgin breast (as evident in the mouse mammary gland), a quiescent state of the epithelial cells is coincident with high levels of p53 mRNA. In response to pregnancy initiation, by contrast, at the end of each luminal epithelia branch, sac-like structures called alveoli differentiate, with a lining of milk-producing cells 'lactocytes', which are active during lactation (Visvader and Stingl, 2014). The modelling of alveoli 'sacs' *in vitro*, referred to as 'acini', has exposed that their proper hollowed sculpting is intrinsically subject to p53-induced apoptosis (Zhang et al., 2011). Furthermore, proper mammary gland ductal morphogenesis requires the strict regulation of p53 to ensure proper stem function with asymmetric cell division (Aparicio and Eaves, 2009). These important findings are beginning to refine our understanding of p53 in mammary fat pad development. New studies using inducible models under appropriate promoters are warranted to avoid the non-Mendelian genetics associated with early studies of whole body p53 knock-out mice, in which female survival was the exception (Haupt et al., 2013), predictive of abnormality (and possibly the reason for the failure to observe a significant mammary phenotype in the early mouse studies) (Jones et al., 1995).

Excess p53 activity in the mammary gland has been associated with reduced growth. Intriguingly, hormone stimulation (including oestrogen and progesterone) proved capable of overcoming the growth inhibition imposed by elevated p53 levels (Gatza et al., 2008). It is tempting to speculate a role for MDM2 in this relief of p53, although this awaits experimental confirmation.

During lactation, mammary epithelial cells are stimulated by pro-survival stimuli while in response to weaning, breasts undergo involution with significant apoptosis and size reduction. P53 is active during the apoptotic phase of involution (Jerry et al., 1998). Proper regulation of p53 throughout these stages is required. It is speculated that appropriate relief of p53 from the restraint of the MDM family is necessary to allow these processes to occur (Baxter et al., 2007; Watson and Kreuzaler, 2011).

In addition, the p53 family member, p63, is pivotal for proper mammary gland development (Mills et al., 1999; Yang et al., 1999) and is vital for survival and maintenance of mammary epithelial cells that arise during pregnancy (parity), persist during involution and provide progenitors for ducts and alveoli in additional pregnancies. P63 in this select population inhibits apoptosis driven by p53, but also contributes to cancer protection associated with parity (Yallowitz et al., 2014). MDM2 interaction with p53 family member p63 has been identified (Stindt et al., 2015), and it is fair to say that the regulation of the p53 family functions by the MDM family remains understudied in breast tissue. However, since the importance of these molecular interactions has been shown in other tissues, it is relevant to discuss the contribution of p53 and its family members to proper breast morphogenesis in this MDM family review. Together, these anti-oncogenic functions of normal levels of MDM2 predict the risk of inappropriate levels for cancer.

Disruption of the MDM family occurs in BrC

As introduced, p53 and the MDM family are in a finely tuned interactive, regulatory loop, and interference with their normal function compromises this vital dynamic. Specifically, deregulation

of their levels or corruption of their integrity has important ramifications for cancer.

MDM2 overexpression poses a BrC risk

Ductal hyperplasia development in transgenic MDM2-overexpressing mice is direct evidence of the oncogenic capacity of MDM2 in mammary tissue. Disruption of the normal replicative divisions associated with cell cycle in these duct cells was attributed to MDM2 overexpression. Approximately 16% of these transgenic mice developed mammary tumours at late age (Lundgren et al., 1997). Elevated MDM2 protein levels are also associated with human BrC.

MDM2 protein levels have been identified as an independent prognostic biomarker in human BrC [Park et al., 2014] and reference within it]. Abnormally high levels of MDM2 protein have been measured in at least 1/3 (38%) of human BrCs, which could not be explained by gene amplification alone (Yu et al., 2014).

A rational explanation of the tumourigenic selection of high MDM2 protein levels in a wt p53 context is the need to evade its growth suppression. A number of mechanisms have been defined that can contribute to MDM2 hyperactivity, and this appears most relevant in a wt p53 context (Figures 1 and 2).

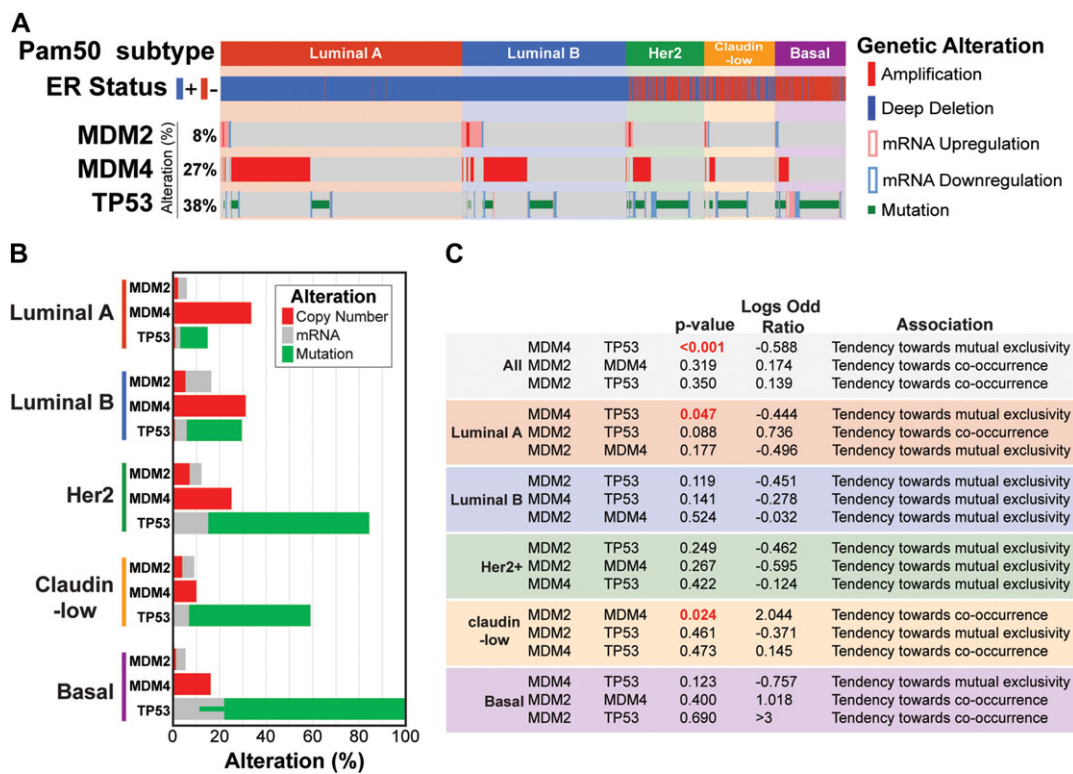


Figure 1 The genomic profiles of 1826 BrC patients in the METABRIC data set (Pereira et al., 2016) based on the five major BrC subtypes. (A) Shown are the somatic mutations (green), copy number deletions (blue), amplifications (red), and mRNA alterations for MDM2, MDM4, and TP53. (B) Similar to A, shown are the percentage and types of alterations for MDM2, MDM4, and TP53. (C) A table showing the logs odds for significant (red) mutually exclusive alterations and co-occurrence alterations for MDM2, MDM4, and TP53 across the five major BrC subtypes. Notably, MDM4 is significantly overexpressed in wt TP53 in BrC, especially the luminal A subtype, while MDM2 and MDM4 are commonly overexpressed together in the claudin-low subtype. All data are derived from cBioPortal (Gao et al., 2013) (<http://www.cbioportal.org>).

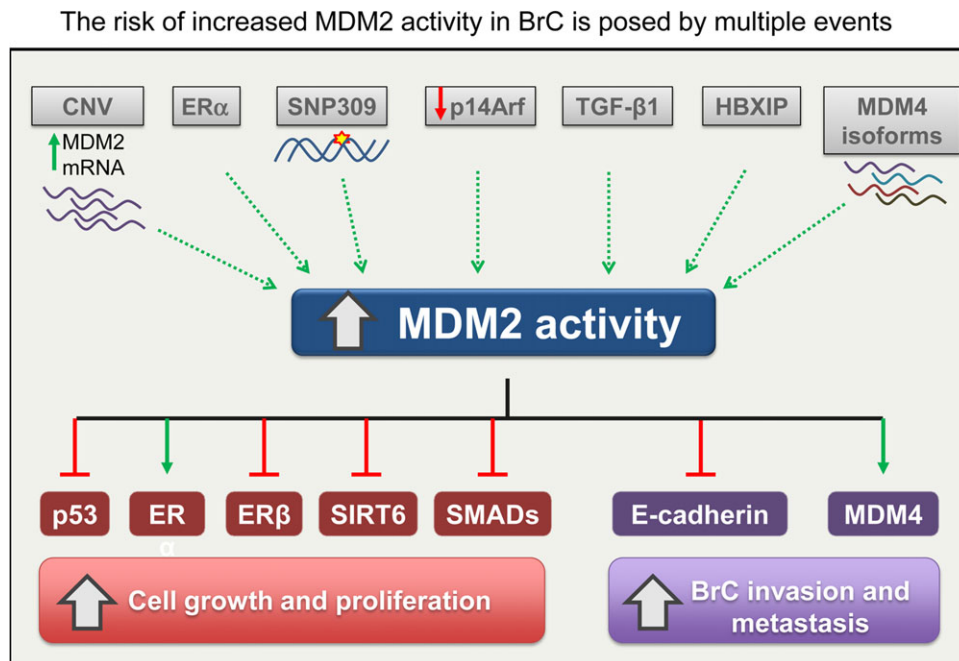


Figure 2 MDM2 activities are frequently elevated in BrC due to multiple events. Key risks for BrC include overexpression of MDM2 mRNA and CNV, mislocalization due to reduced p14ARF sequestration, ER α SNP309, the hepatitis B molecule HBXIP driving MDM2 transcription, TGF- β 1 induction of SMAD3 causing increased MDM2 transcription, altered expression of MDM2 isoforms, etc.

Firstly, elevated MDM2 protein levels reported in BrC resulting from changes in MDM2 mRNA expression and copy number are a rare occurrence in ER-positive BrC (Figure 1A). Overexpression of MDM2 was, however, most evident in ER+ and PR+ luminal BrC (Figure 1B), generally coincident with wt p53 (Yu et al., 2014). MDM2 coding alterations were also generally at low incidence in the METABRIC BrC data sets, although to varying degrees across the subtypes (Figure 1B). Similar findings of MDM2 amplification across the BrC subtypes were also reported by others (Cancer Genome Atlas, 2012).

A second contributor to increased MDM2 activity in human BrC relates to cellular location. While MDM2 is normally sequestered into the nucleus under the directive of p14Arf (in humans, p19Arf in mice), in many human BrCs, it is freed from this spatial constraint, as p14Arf is at abnormally low levels. Low p14Arf levels are due to its deletion, loss of heterozygosity, or promoter hypermethylation (Ozenne et al., 2010).

Thirdly, increased MDM2 levels can result from genetic modifications of MDM2 regulators. The finding that ER α can promote MDM2 levels is consistent with this elevation. Specifically, ER α SNP309 (G/G) has been associated with higher cancer susceptibility, including BrC in pre-menopausal women (Hu et al., 2007). The co-occurrence of an additional SNP285C, exclusively in Caucasian women, is attributed to lower BrC risk [(Knappskog et al., 2011) and references within it]. Importance of SNP309 in familial BrC remains unclear. Association between cancer susceptibility and increased incidence of SNP309 (G/G) was also deduced in Li-Fraumeni families (Ruijs et al., 2007), both carriers and non-carriers of *TP53* mutations. In other familial BrC

families, the importance of SNP309 was less clear-cut and begs further study (Wasielowski et al., 2007; Nechushtan et al., 2009). Also, amplification of the gene encoding ER α (*ESR1*) has been noted in ~20% of BrCs in a cohort of 2000 biopsies; however, unlike the SNPs, this was not associated with MDM2 amplification and did not combine with its prevalence to elaborate prognosis or predict treatment (tamoxifen) outcome (Holst et al., 2007). Further stratification combining the parameters of *ESR1* amplification together with the SNPs would seem warranted for assessing compounding BrC risk.

Fourthly, MDM2 levels are also elevated in hepatitis B infection. Interestingly, the hepatitis B virus X-interacting protein (HBXIP) was found to increase MDM2 levels in BrC, with MDM2 promoter activation executed by direct binding to p53 at its P2 site (Li et al., 2015). Beyond these phenomena, alternative post-translational events are needed to explain the elevated MDM2 protein levels that are not a result of increased mRNA transcript in BrC. Fifthly, in late stage metastatic BrC, transforming growth factor (TGF)- β 1 signalling can induce MDM2 transcription through its second promoter by the induction of Smad3 (Araki et al., 2010).

In addition to overexpressed MDM2 posing a risk for BrC due to its capacity to destroy wt p53, its additional oncogenic capacities compound the danger of its overexpression. First, MDM2 elevates the levels of the tumour-promoting ER α (Brekman et al., 2011; Swetzig et al., 2016). Second, in contrast, MDM2 diminishes the levels of ER β , an emerging tumour suppressor capable of reducing the migration potential of BrC cells by increasing the expression of adhesion proteins. In response to

AKT signalling, MDM2 forms a complex with ER β and the coactivator CBP, where it ubiquitinates and promotes the degradation of this hormone receptor. MDM2 depletion rescued ER β and inhibited BrC cell proliferation, predicting the value of therapeutic intervention (Sanchez et al., 2013). Third, elevated MDM2 E3 ligase activity can drive resistance to therapies through a number of pathways. MDM2 was shown to ubiquitinate and degrade SIRT6, a major tumour suppressor. This was found to contribute to Trastuzumab resistance in Her2+ BrC (Thirumurthi et al., 2014). Fourth, in a number of BrCs not responsive to TGF- β , MDM2 overexpression in association with MDM4 affected the transcriptional activity of SMAD family of proteins and thereby blocking TGF- β mediated growth arrest. This contributed to TGF- β resistance (Sun et al., 1998; Yam et al., 1999). Fifth, elevated MDM2 levels were identified to promote E-Cadherin ubiquitination and degradation that in turn drove cancer cell invasiveness (Yang et al., 2006). Sixth, increased expression of matrix metalloproteinase 9, a recognized enhancer of invasion and metastasis, increased with MDM2 overexpression in BrC cells (MCF7 and MDA-MB-231). Similar correlation in expression levels was also identified in BrC samples, specifically in invasive ductal carcinoma IDC samples (Chen et al., 2013). These studies demonstrate the potency of deregulated MDM2 to drive invasion and metastasis in BrC in p53-independent pathways.

MDM2 levels inversely correlate with TP53 mutation in BrC

In contrast to a wt p53 context, *TP53* mutation and *MDM2* amplification emerge with mutual exclusivity across the BrC subtypes (Figure 1C), which is consistent with literature findings (Sheikh et al., 1993; Quesnel et al., 1994; Marchetti et al., 1995; Yu et al., 2014). Critically, accumulation of mutant p53 is a basic prerequisite for its oncogenic neomorphic properties, termed gain of function (Brosh and Rotter, 2009). *TP53* mutation is a risk for aggressive BrC. Sporadic *TP53* mutation is particularly frequent in late stage BrC, where it promotes metastasis (Powell et al., 2014). Also BrC is the most frequent cancer resulting from germline *TP53* mutation in Li-Fraumeni families (Gatza et al., 2008).

Not only does mutated *TP53* lack the capacity to transcriptionally activate the *MDM2* gene, but evasion of MDM2 amplification avoids the risk of mutant p53 degradation (Haupt et al., 1997). This is consistent with the lower MDM2 protein levels measured in basal-like BrC compared to luminal and Her2+ subtypes as we recently reported (Haupt et al., 2015). The critical deregulation of the p53/MDM2 pathways during malignant transformation has been ably modelled by the transition of the untransformed MCF10A harbouring wt p53 to a transformed counterpart, where high levels of MDM2 are an indicator of oncogenic potential (Su et al., 2011; Munne et al., 2014).

Furthermore, p53 isoforms have been described to contribute to BrC (Khoury and Bourdon, 2011), which adds an additional level of complexity. As mentioned in a healthy cell context, MDM2 exists as isoforms and their relative levels dictate their activity towards p53. Diabolically for cancer, interference with the capacity of MDM2 to degrade p53, through alteration of

MDM2 isoform levels, can promote the accumulation of not only wt p53, but also its mutant form (Zheng et al., 2013). Also, p53 isoforms differ in their affinity for p53-responsive elements (RE) in the promoters of their target genes. Wt p53 has higher affinity than the p53- β isoform for the p53 RE in the promoter of MDM2 (Bourdon et al., 2005). Pertinent to this review, abnormal p53 interferes with proper MDM family regulation.

Oncogenic MDM2 isoforms promote BrC

As MDM2 exists in a number of isoforms, it has been questioned whether these have different functional capabilities. The most frequently expressed splice variant in cancers including BrC is MDM2-B (Matsumoto et al., 1998). However, as mentioned above, it is also present in normal breast tissue (Gudas et al., 1995; Lukas et al., 2001). MDM2-B is unable to bind to p53, but can bind to MDM2-FL and sequester it in the cytoplasm. In normal cells, this increases wt p53 activity (Evans et al., 2001). By analogy, it was speculated that this isoform is tolerated in cancer because it also protects mutant p53 (Bartel et al., 2002), thereby conferring an oncogenic advantage (Figure 2).

High levels of MDM4 promote BrC

The capacity of MDM4 to inhibit p53 transcriptional activity, even in the absence of E3 ligase activity (Marine et al., 2007), together with the enhanced capacity of MDM2 to ubiquitinate p53 in the presence of MDM4 (Linares et al., 2003), predicts that MDM4 overexpression poses a cancer risk. In addition, p53-independent oncogenic activities have been associated with MDM4. MDM4 was shown to promote pRb degradation by MDM2, with consequent E2F1 activation of the cell cycle (Zhang et al., 2015). Also, the capacity of MDM4 to suppress p21 (Jin et al., 2008) and p27 (de Lange et al., 2012) is consistent with elevation of their levels in response to MDM4 depletion (in certain cancer cell types). ER α has also been reported to promote MDM4 levels, apparently in a p53-independent manner (Swetzig et al., 2016), indicating its impact across the MDM family.

It is not surprising then, that elevated levels of MDM4 were measured in BrCs (65%), accompanied in the majority of cases by low-level p53 (62%), which was apparently wt, as suggested by an absence of allelic loss and no increased protein detection. Yu et al. (2014) identified significantly increased *MDM4* gene amplifications (56.5%) by fluorescence *in situ* hybridization (FISH), which contrasted with the far more modest levels (5%) previously described (Danovi et al., 2004) and our findings (Haupt et al., 2015). The discrepancy between these findings is apparently due to what is considered amplification, where the former included low-level amplifications (Yu et al., 2014). These copy number changes were most evident in luminal and Her2+ BrC subtypes (Figure 1). An extreme instance is evident in the luminal BrC line MCF7, which maintains wt p53 at undetectable protein levels, in the presence of extreme amplification of the *MDM4* gene (Danovi et al., 2004). The selection is consistent with its capacity to promote the E3 ligase activity of MDM2 and render p53 inactive in these cells.

In triple negative breast cancers, which are largely p53-mutant, we reported high MDM4 levels that are not a consequence of gene amplification (Haupt et al., 2015). These high MDM4 protein levels generally inversely correlate with low MDM2 levels. Selection for elevated MDM4 in this context is consistent with its additional wt p53-independent functions.

Alternatively spliced MDM4 isoforms contribute to oncogenesis

The mechanism for high MDM4 levels in the absence of significant gene amplification has been the subject of considerable study in BrC (Lenos et al., 2012). MDM4 RNA splice variants that are generated by an exon skipping process have been deduced to have distinct stability. MDM4-FL is stable, while a shorter form MDM-S, which lacks exon 6 and is short-lived, is not. A switch from MDM-S to MDM-FL contributes to MDM4 oncogenesis (Figure 3).

Elegant studies in melanoma identified that interruption of the spliceosome by abnormal activity of oncogenic SRSF3 promoted the inclusion of exon 6, leading to generation of MDM-FL, with consequent oncogenic consequences (Dewaele et al., 2016). It remains to be studied whether the aberrant splicing evident in BrC is also due to disruption by SRSF3. By extrapolation from melanoma, this altered splicing mechanism offers the potential for therapeutic intervention.

MDM4 SNPs have ramifications for BrC susceptibility

At another level, MDM4 SNPs have also been linked to BrC susceptibility. In synergy with MDM2 SNP309 G/G, MDM4 SNP 34091 A/A associated with enhanced oncogenesis. In contrast, in association with the same MDM2 SNP309, MDM4 SNP 34091 C/C was associated with reduced risk in Norwegian BrC patients (Gansmo et al., 2015). MDM4 SNP (SNP rs1563828) on intron 10 was associated with the early onset of disease, particularly in ER-negative BrC (Kulkarni et al., 2009). In the Askenazi Jewish population, additional SNPs have also been linked to early onset of BrC (Atwal et al., 2009).

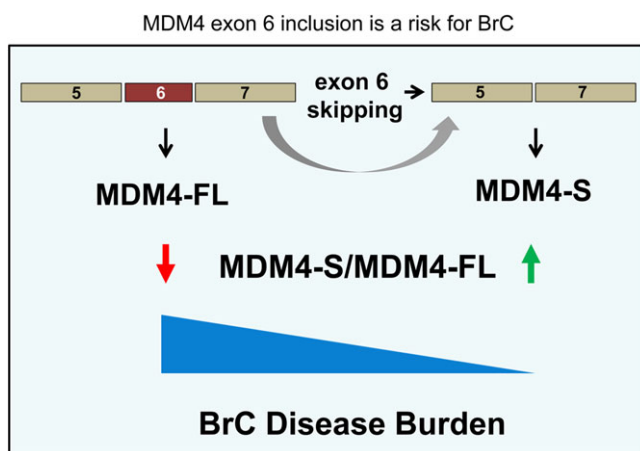


Figure 3 Alternative splicing of MDM4 is a risk for BrC development. MDM4 exon 6 inclusion diverts generation of an degradation-prone isoform MDM4-S to a stable MDM4-FL isoform that is associated with BrC development (Lenos et al., 2012).

MDM2 and immunity

The link between the immune system in cancer is slowly emerging and a link to the MDM family has largely been ‘by association’ with p53, rather than elaborated with extensive experimentation.

A major exception is the report of the capacity of MDM2 to inhibit T cell activation by promoting the ubiquitination of NFATc2. More specifically, CD4+ T cells, but not CD8+ T cells, are subject to this regulation, and this is in a p53-independent manner (Zou et al., 2014). In a fascinating reciprocal regulation, T cell activation promoted the degradation of MDM2, possibly by stimulating self-ubiquitination, in contrast to its stability in resting T cells. The stability of MDM2 is controlled upstream by the deubiquitinase USP15. Elevated levels of USP15 stabilize MDM2 and repress T cell activation, predicting the therapeutic relevance of targeting USP15 (Zou et al., 2014).

MDM2 and MDM4 therapies

There are a number of MDM2 and MDM4 inhibitors currently in clinical trials alone and also in combination with chemotherapies or other molecularly targeted therapies (Burgess et al., 2016). None of these are specific to BrC at the current time. A fraction of these trials have pre-specified tumours with wt p53 as an inclusion criterion.

New inhibitors of the MDM family are also in development for clinical application. The majority of these emerging therapies for targeting MDM2 and MDM4 are small molecule inhibitors; however, the majority bind preferentially to MDM2 (Li et al., 2003). One major consideration in developing this class of therapies in BrC is the varied amplification levels of MDM2 and MDM4 and incidence of *TP53* mutation across different BrC subtypes. It is unclear at this stage how these biomarkers predict for response to MDM2 inhibitors. Compared to MDM4 amplification, BrC has a relatively low incidence of MDM2 amplification, with the highest incidence in the luminal B subtype (13%). Unlike the small molecule inhibitors of MDM2 in clinical development, ALRN-6924 (Aileron Therapeutics) is a stapled peptide designed to disrupt p53 interaction with both MDM2 and MDM4 and currently in phase I/II clinical trials in solid and haematological malignancies (Clinicaltrials.gov identifier NCT02264613 and NCT02909972). This may have more clinical potential in BrC, as MDM4 is more frequently amplified compared to MDM2.

Another important consideration is the toxicity profile of this class of drugs, particularly as it overlaps with toxicities associated with commonly used chemotherapies used in BrC. RG7112 and RG7338, which are both MDM2 inhibitors, have been associated with high rates of gastrointestinal and haematologic toxicity, the latter showing the most common dose-limiting toxicity (Ray-Coquard et al., 2012; Siu et al., 2014; Andreeff et al., 2016). Long-term toxicity is, however, currently unknown.

Conclusions

The MDM family plays critical roles in breast development, function, and protection from cancer. Their best-characterized cancer prevention activity is regulating appropriate responses of

the major tumour suppressor p53. The p53 suppressive capabilities of the MDM family are the likely explanation for their overexpression in many BrCs. However, the MDM family also has p53-independent activities that may become tumorigenic. The recognition of the oncogenic function of this family in BrC is leading to the development of tailored drugs with application to BrC and beyond.

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