Title

PGRMC1 phosphorylation status and cell plasticity 2: genomic integrity and CpG methylation

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ABSTRACT

Background: During vertebrate embryology the state of animal cell differentiation is determined by several mechanisms, including heritable but plastic patterns of genomic and histone methylation. Cancer is a disease related to loss of terminal differentiation. Progesterone receptor membrane component 1 (PGRMC1) is a protein that has been implicated in cancer biology and poor patient outcomes. It can be over-expressed in cancers, and exist in alternative states of phosphorylation. A motif centered on PGRMC1 Y180 was evolutionarily acquired in animals by the common ancestor of Cnidaria and bilateral animals, concomitantly with an embryological organizer of gastrulation associated with the induction of systematically regulated epigenetic genome methylation.

Results: Here, we show that manipulation of PGRMC1 phosphorylation by mutagenesis results in altered cell metabolism, genomic stability, and CpG methylation. Hypothesizing that PGRMC1 phosphorylation status affects genome methylation, we discovered that each of several mutants elicited distinct patterns of CpG methylation. Mutation of Y180 led to increased overall hypermethylation levels, accompanied by hypomethylation of selected promoter- and cell-type enhancers, reminiscent of the hypermethylated status of embryonic stem cells. Pathways enrichment analysis suggested modulation of processes related to animal cell differentiation status and tissue identity, as well as cell cycle control and ATM/ATR DNA damage repair regulation. We detected PGRMC1 phospho-status-dependent differential rates of genomic mutation after thirty culture passages.

Conclusions: In companion manuscripts we show that 1) these cell states are associated with dramatic effects on protein abundances, cell and mitochondrial morphology, and glycolytic metabolism, and 2) that Y180 has been evolutionarily conserved since its simultaneous appearance with the animal gastrulation organizer. These combined studies suggest that PGRMC1 phosphorylation status, as controlled by ill-defined signaling

processes affected by phosphorylation site mutants, modulates cellular plasticity by affecting mechanisms relevant to early embryological tissue differentiation, which should be highly relevant to disease.

KEY WORDS

epigenetics, genomic sequence, hyperspectral autofluorescence, organizer, embryology, metabolism, cell death, cytochrome P450, steroid biology

BACKGROUND

Progesterone (P4) Receptor Membrane Components 1 (PGRMC1) is a cytochrome b_5 (cytb5) protein and a member of the membrane-associated P4 receptor (MAPR) family with a plethora of cellular functions. These include some cytb5-typical functions that predate or are potentially ancient in eukaryotes (regulation of heme synthesis [1], cyP450 (cytochrome P450) interactions [2-4], sterol metabolism [5-7]), some that evidently arose in eukaryotes (membrane trafficking [reviewed by: 8, 9], cell cycle regulation at the G0/G1 checkpoint [8, 10, 11], mitotic/meiotic spindle association [12-14]), and clearly specialized metazoan functions including e.g., fertility, embryogenic axon guidance, and membrane trafficking associated with synaptic plasticity [reviewed by: 8, 15]. CyP450-interactions [2] conspicuously feature PGRMC1 regulation of the most conserved eukaryotic cyP450 (lanosterol 14-alpha demethylase, CYP51A) to modify the first sterol (lanosterol) from yeast to mammals [6, 7, 16]. The protein is accordingly found in diverse subcellular locations, including endoplasmic reticulum/perinuclear membrane and Golgi apparatus [17, 18], at the cell surface [19, 20], mitochondria [1], and the nucleus, where it localizes to nucleoli [21]. It is strongly associated with sterol biology (reviewed by: [5, 16]), and a complex between PGRMC1, the low density lipoprotein (LDL) receptor (LDLR) and the sigma-2 receptor transmembrane protein 97 (TMEM97) regulates the rapid internalization of LDLR [22].

Indeed, PGRMC1-associated membrane trafficking determines the cell surface localization of a number of different proteins including e.g., epidermal growth factor receptor [23], insulin receptor and glucose transporters GLUT-1 and GLUT-4 in A549 lung cancer cells [24], glucagon-like peptide-1 (GLP-1) receptor complex in pancreatic beta cells [25], synaptic vesicles in the central nervous system [19], and membrane progestin receptor α (mPRα) in rat granulosa cells [26] and maturing zebrafish oocytes

[27]. The translocation of transmembrane proteins such as membrane receptors to the cell surface could underlie much of the biology attributed to PGRMC1 [28].

PGRMC1 has attracted considerable attention in the field of cancer biology, e.g.: [29-33]. Suppression of apoptosis, DNA damage protection, and resistance to chemotherapy are among the well-observed PGRMC1 roles in cancer (reviewed by [15]), and it has been implicated in elevated cancer rates of patients undergoing progestogen therapy [31, 34-36]. Anti-apoptotic activity of PGRMC1, especially to treatment with the topoisomerase II inhibitor doxorubicin, has been reported by several studies [37, 38]. This activity was linked to P4- and PGRMC1-dependent survival responses [39].

It remains unclear how much this P4-responsiveness involves the membrane trafficking of actual P4 receptors to the cell surface, or to what extent PGRMC1 itself may mediate its own P4 response (reviewed by [8, 15]). PGRMC1 undeniably confers P4 responsiveness to cells of the reproductive [40] and the central nervous systems [41]. A D120G heme-binding incapacitated mutant also exhibited reduced P4-responsiveness, suggesting independence from cyP450 activation [39]. PGRMC1 associates with the CYP2D6 and CYP3A4 cyP450 enzymes responsible for doxorubicin hydroxylation and inactivation [42], and this activity required the heme-chelating residue Y113. It was concluded that heme-mediated dimerization was required [42]. However, since Y113 is a phosphorylated residue at a consensus membrane trafficking motif, the effect may instead have required tyrosine phosphorylation (and perhaps membrane trafficking) [43], which remains unresolved.

PGRMC1 is induced by a variety of agents causing DNA damage, including doxorubicin [15], and its yeast Dap1 (*d*amage-*a*ssociated *p*rotein 1) homologs are associated with

DNA damage response [6, 44]. Heme-binding and cyP450 interactions are thought to be required for DNA damage response [6, 15].

Ahmed et al. showed that A549 cells treated with so-called PGRMC1 inhibitor AG-205 exhibited G1 arrest and an increase in sub-G1 (apoptotic) cells. Also, AG-205 treated cells showed a reduction in the S and G2/M stages [28]. Note that AG-205 is often cited as a PGRMC1-specific inhibitor. While it certainly affects PGRMC1-dependent function, it was developed against a plant MAPR protein [45]. There is no evidence that it is any more specific to PGRMC1 than to PGRMC2, other MAPR family members Neudesin or Neuferricin, or indeed that it does not exert effects involving non MAPR proteins. Claims related to "PGRMC1-specific inhibitor AG-205" should be treated circumspectly.

The PI3K/Akt pathway is affected by PGRMC1 in MCF-7 cells by PGRMC1 overexpression [46] or overexpression of phosphorylation site mutants [18] and in an accompanying paper we show that Y180 is required for PI3K/Akt activity [47]. PGRMC1 was differentially phosphorylated between estrogen receptor-positive and negative breast cancers [18]. Human PGRMC1 contains predicted binding site motifs for Src homology 2 (SH2) and Src homology 3 (SH3) domain-containing proteins, with several other phosphorylation sites at S57, T178 and S181 being thought to additionally regulate these sites [43]. S57 and S181 are both consensus casein kinase 2 (CK2) sites, and thought to sterically interfere with protein interactions at adjacent proline-rich SH3 target motif and SH2 target motif based upon Y180, respectively [43]. Both are phosphorylated in serum-starved HEK293 cells [48]. Simultaneous mutation of both PGRMC1 phosphorylation sites S57 and S181 permitted MCF-7 breast cancer cells to survive otherwise lethal redox stress [18]. These and an SH2 target motif based on Y139 are mutually juxtaposed on the folded protein surface, forming a potential proximity-stimulated signaling platform [16,

43]. Thus, PGRMC1 represents a potential integration point and effector of many cell signaling pathways responsible for growth and proliferation [8, 16, 28, 40].

PGRMC1 was found to be induced in ductal carcinoma *in situ* breast lesions in the hypoxic zone surrounding the necrotic core, at the time and place that tumor cells must adopt glycolytic anaerobic metabolism. Its induction preceded that of Hif-1-induced GLUT4 glucose transporter in some cells [18]. This prompted speculation that PGRMC1 may be involved in the Warburg effect [8], or aerobic glycolysis: a metabolic state where cells predominantly employ glycolytic glucose metabolism even in the presence of oxygen, and which is associated with stem cell-like biology [49]. Sabbir recently demonstrated that PGRMC1 exists as different post-translationally modified isoforms (phosphorylation, sumoylation, ubiquitination), which alter abundance dynamically in response to P4 in HEK293 cells. This was associated with a P4-dependent increase in aerobic glycolysis resembling the Warburg effect [48].

PGRMC1 association with transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF) inhibits the activation of genes associated with mitotic signaling with potential cancer relevance [48, 50-52]. Vertebrate TCF/LEF transcription factors belong to a family of isoforms which are the major targets of Wnt signaling. This features prominently in animal embryology as well as in maintaining stem cell status in adult and embryological cells. Glycogen synthase kinase 3-β (GSK3-β) phosphorylation of β-catenin targets it for polyubiquitination and degradation. In canonical Wnt signaling this degradation is inhibited after Wnt activation, so that β-catenin can then translocate to the nucleus, where it interacts with TCF/LEF to convert TCF/LEF-mediated repression into transcriptional activation [53-55]. In human embryonic stem cells (hESCs), a cell culture model closely paralleling early embryology, PGRMC1 expression is associated with

repression of Wnt signaling, and inhibition of differentiation (maintenance of pluripotency) [56]. Interestingly, that cell system is one of the conditions where the PGRMC1 cytb5 domain is expressed lumenally, rather than conventional cytoplasmic topology, being extracellular on the cell surface [20]. PGRMC1 is also found extracellularly in neuronal synapses [19], and in A543 lung cancer cells, where it is extracellularized by the exosome pathway [57].

As a target of Wnt signaling, TCF/LEF appeared very early in animal evolution, being absent from the protist Choanoflagellate sister group to metazoans, but present in sponges [53]. By the time the common ancestor of Cnidaria (corals, jellyfish, etc.) and Bilateria (bilaterally symmetrical animals) evolved, embryological gastrulation initiated from the animal pole under control of Wnt/β-catenin signaling, inducing an axial organizer whose vertebrate descendant is known as the Spemann-Mangold organizer. This initiates the orchestrated series of events where specific gene programs are activated by transcription factors that trigger differentiation into specialized cell types [53, 58-60]. Animal embryology relies heavily on epigenetic histone and genomic methylation to embryologically direct cell plasticity between generations and in response to stimuli to maintain the differentiation status of cells [61]. Sperber et al. have reported that hESC pluripotency is maintained epigenetically by the activity of the nicotinamide-N-methyltransferase (NNMT) pathway, which globally depletes cell methyl donor levels, resulting in reduced methylation and elevated expression of genes including Wnt pathway genes [62]. Note that hESC pluripotency is affected by both PGRMC1 via a Wnt-dependent mechanism [56], and by global epigenetics [62], which are therefore potentially related.

In an accompanying paper [63] we show that the combination of PGRMC1 Y139 and Y181 was first acquired by the Cnidaria/Bilateria common ancestor, coincident with the evolution of the ancestral embryological gastrulation organizer [60], and that these residues are strongly conserved in bilaterian animals [63]. The SH3 target motif was acquired during mammalian evolution [64].

In another accompanying paper [47], we found that mutating hemagglutinin (HA)-tagged PGRMC1 (PGRMC1-HA) S57 and S181 phosphate acceptor amino acids, which are thought to regulate protein interaction motifs, induced dramatic effects in MIA PaCa-2 (MP) pancreatic cancer cells. This double mutant (DM) exhibited elevated PI3K/Akt activity, altered actin cytoskeleton proteins, and elevated migration rates relative to MP cells expressing wild-type (WT) PGRMC1-HA protein. Additional mutation of Y180 in a triple mutant (TM) attenuated PI3K/Akt activity and mouse xenograft tumor growth. Pathways enrichment analysis of a proteomics study suggested broad effects relevant to cancer biology, including effects on glucose metabolism and mitochondrial form and function that were suggestive of plastic cellular reprogramming. Here, we characterize those cells further considering effects on metabolism, genomic stability and epigenetic plasticity.

RESULTS

Hyperspectral autofluorescence reveals PGRMC1 phosphorylation state-dependent metabolic differences

To investigate the differences between cells we investigated cell metabolism. Cells were subjected to preliminary hyperspectral autofluorescence imaging: a non-invasive analytical technique [65]. Distinct spectral signatures of endogenous auto-fluorescent molecules [66, 67] can provide valuable information about intracellular metabolic state

[68]. As a first step towards demonstrating discrimination of cell conditions based on spectral information, eight informative spectral features that discriminated the cells were chosen (Figure 1A and Table 1). The cells in each condition could be clearly distinguished using the autofluorescence of their endogenous metabolites. A pairwise linear discrimination analysis using the same spectral features indicated that all cell conditions were highly significantly separated from each other on the basis of endogenous autofluorescence (Figure 1A).

Figure 1B shows autofluorescence differences in spectral channel 3, thought to reflect flavin emission [68, 69]. All cells over-expressing PGRMC1-HA proteins exhibited reduced levels, with DM and TM the lowest (Figure 1B). We also noted significant differences observed between cell lines for autofluorescent emission at 700 nm (Figure 1C), in a wavelength range at which heme-containing proteins and other porphyrins contribute strongly to autofluorescence [70]. Note that PGRMC1 not only binds heme but is involved in heme synthesis [1]. Heme itself does not emit fluorescence but leads to fluorescence quenching, however, various heme-containing proteins are fluorescent [70]. Although the actual identities of these and fluorescent species contributing to many hyperspectral channels remain unknown, several unidentified parameters also significantly discriminated between the metabolites present in the different cell lines, such as e.g. the ratio of channel 3 to channel 12 (Figure 1D, Table 1). These results indicated that PGRMC1 phosphorylation status dramatically alters cell metabolic state.

PGRMC1 phosphorylation mutants did not affect P4 or AG-205 responses

We were interested in whether DNA mutation rates may have been related to cyP450 interactions related to the P4-dependent protection against cell death, or to the mechanism of AG-205-indiced cell death. MP cells have not previously been reported as being P4-

responsive. Consistent with previously reported PGRMC1-dependent anti-mitotic effects of P4 in Ishikawa endometrial cancer cells [38] and MDA-MB-231 breast cancer cells [71], incubation of cells in 1μM P4 retarded cell proliferation of all cells over-expressing a PGRMC1-HA protein (WT, DM or TM), but not of MP cells relative to non-P4-treated control cells (Figure 3A). When cells pretreated with or without P4 for 1 hr were co-incubated in the presence doxorubicin (Dox), in the absence of P4 then WT, DM and TM cells were more susceptible to Dox-induced death (Figure 3B-C, white data points and boxes), however these cells exhibited greater P4-dependent protection against Dox-induced death (Figure 3B-C, shaded data points and boxes). As reported previously for Ishikawa cells [38], the 1hr pre-incubation with P4 was required to observe these protective effects of P4 (data not shown). There was no significant protective effect of P4 on cell survival for MP cells (Figure 3C). Notably, there were no significant differences in P4-dependent protection between WT, MP or TM cells. There was also no difference in the sensitivity of any of these cells to AG-205-induced cell death, with essentially superimposable dose response curves, and a half maximal lethal effective concentration (EC_{50}) of approximately 32μM AG-205 (Figure 3D-E). Therefore, although we observe PGRMC1-HA-dependent response to P4, this response is apparently unaffected by the influence of our PGRMC1 phosphorylation mutants. We conclude that the dramatic effects of altered PGRMC1 phosphorylation status observed in this and the accompanying manuscript [47] are due to different and newly described functions of PGRMC1, unrelated to the mechanism of P4-induced vitality or AG-205-induced death.

PGRMC1 phosphorylation status affects cytoplasmic redox status

Naphthalimide-flavin redox sensor 1 (NpFR1) is a fluorophore analogous to NpFR2 [47, 72], except that it is localized to the cytoplasmic compartment of cells [73]. To further explore metabolic state, cells were treated with NpFR1 and fluorescent state was assayed

by flow cytometry to reveal two cell populations: one with less oxidized NpFR1 and one with more oxidized NpFR1 (Figure 2A). The bimodal fluorescent peaks of Figure 2A represent cell populations with different levels of cytoplasmic oxidation/reduction which have not been further characterized. All PGRMC1-HA-expressing cells exhibited the majority of cells in a more oxidized peak. TM (9.4%) and WT (17.7%) cells both exhibited less cells in the lower oxidized fraction (Figure 2A,B, black arrows). For TM cells, which had most cells in the more oxidizing cytoplasmic population, the degree of NpFR1 oxidation in that fraction was significantly lower than for all other cells (Figure 2A,C, white arrow). Higher cytoplasmic oxidative levels in TM cells suggested potential effects on genomic mutation rates.

PGRMC1 phosphorylation status affects pathways associated with replication, cell cycle, and mitogenic signaling

In an accompanying paper [47] we presented only highly significant differential WebGestalt pathways with Benjamini-Hochberg adjusted *p* (adjP) values <0.001. Several pathways related to cell cycle control and DNA replication were also detected, albeit with weaker significance, including five proteins predicted to be associated with decreased levels of ATM/ATR DNA damage sensing and control in TM cells (Figure 4A), which was interesting to us because of the DNA damage associations of PGRMC1 (see Introduction). Although we could not detect differences in cultured proliferation of any cell lines [47], reverse phase protein array (RPPA) analysis showed elevated levels of activated MKK4, MEK and ERK, as well as phosphorylated retinoblastoma protein (Rb) in TM cells (Figure 4B). This suggests that PGRMC1 Y180 is somehow involved in traversing the G1 checkpoint, which may be related to the reported ability of PGRMC1 and/or PGRMC1 to lower the propensity of cells to enter the G1 and the cell cycle, rather than enter G0, in spontaneously immortalized granulosa cells [10].

PGRMC1 phosphorylation status affects genomic integrity

Altered cytoplasmic oxidative environment, potential perturbations of the ATM/ATR pathways, and a disconnect between G1 checkpoint (Rb phosphorylation) passage and detectable differences in proliferation rates led us to explore the effects of PGRMC1 phosphorylation status on genomic stability. We cultured one cell line each of MP, WT, DM and TM for 30 passages, and sequenced the genomes of each. The PGRMC1-HA constructs were expressed at similar levels after 30 passages (Figure 5A). Relative to the reference human genome sequence, the order of average mutation rates (difference to reference) per chromosome were WT<DM<TM (Figure 5B). Because of large variation in rates between chromosomes, ANOVA results for average mutation rate per chromosome were not significant. However, paired t-test comparisons of average chromosomal mutation rates were highly significant for the individual pairwise genome comparisons. We also calculated unique mutations per MB in each of those cell lines and assessed them once more as rates per chromosome. Once more we observed the relationship WT<DM<TM. In this analysis pairwise post-hoc Kruskal-Wallis *p* values for the individual pairwise comparisons were all highly significant (Figure 5B). This indicates that manipulation of the phosphorylation status of PGRMC1 Y180 can induce genomic instability, to our knowledge a novel finding of potentially great relevance to cancer biology.

PGRMC1 phosphorylation status regulates the NNMT/1-MNA pathway

To further characterize metabolic differences between cells we performed a pilot metabolomics study. The most prominent metabolite that correlated with the sample clustering of proteomics heat map of the accompanying manuscript [47] was 1-methylnicotinamide (1-MNA). The response of the triple quadrupole mass spectrometer

to 1-MNA was linear over the range of 10 ng/mL to 1 μg/mL, and the identity was confirmed by MS/MS and HPLC retention time using 2-methylnicotinamide and N-methylnicotinamide standards (not shown). Levels of 1-MNA were below the limit of quantitation (LOQ) in the MP and DM lines, but were easily quantitated for the WT cells, and significantly elevated above that for the TM cell lines (Figure 6A). 1-MNA is produced by the enzyme nicotinamide-N-methyl transferase (NNMT), which we found to be elevated in TM cells (Figure 6B). NNMT utilizes S-adenosylmethionine (SAM) as a methyl group donor to convert nicotinamide to 1-MNA [74] (Figure 6C). Liquid chromatography/mass spectrometry (LC/MS) quantification revealed lower levels of SAM in DM and TM cells (Figure 6D). Taken together, lower SAM levels were associated with elevated levels of NNMT mRNA.

We established an anti-NNMT sh-RNA-expressing TM cell line by lentiviral transduction and puromcyin stable selection. Anti-NNMT shRNA attenuated NNMT levels more than two-fold (Figure 6E), which led to expected reduced levels of 1-MNA (Figure 6F). Contrary to the hypothesis that increased NNMT activity would reduce SAM levels as proposed for pluripotent hESCs [62], SAM levels were not significantly altered by NNMT attenuation (Figure 6G). We also treated DM cells with 1-MNA, which caused a morphological conversion from predominantly rounded to predominantly elongated form, suggesting that NNMT activity may contribute to the morphological differences between elongated MP and WT cells on the one hand and rounded DM and TM cells on the other. Other mutants showed no significant change in cell morphology upon 1-MNA treatment (not shown).

PGRMC1 phosphorylation status regulates genomic methylation

Because of the reported effects of NNMT activity on global methylation levels [62], we assayed genomic CpG methylation levels using the EPIC array featuring >850,000 probes. Hierarchical clustering of samples displayed striking separation of the cell groups, suggestive of highly divergent patterns of methylation among the cell types. All independently derived stable cell lines of each PGRMC1 state clustered closely to each other, and distant from other cell conditions (Figure 7A). The third MP replicate (MP-3) was slightly divergent from replicates MP-1 and MP-2 due to a lower overall methylation detection reflecting a poorer sample quality.

The divergent methylation properties of the cell types translated through to differential methylation assessment where we observed a highly significant number of differentially methylated probes (Figure 7B). In the TM/DM comparison (differ by Y180 state) we observed a shift towards hypermethylation that was not as pronounced in WT/MP (differ by transfection of pcDNA3-1_PGRMC1-HA and hygromycin stable selection) or DM/WT (differ by PGRMC1-HA S57/S181 status). We then considered the percentage of differentially methylated probes which were hypermethylated in each comparison and noticed a clear increase in the progression from WT/MP, DM/WT to TM/DM (Figure 7C). While the WT/DM effect could be due to both PGRMC1-HA expression and/or the stable antibiotic selection process, the differences between DM/WT and TM/DM were due solely to PGRMC1 mutation status. The overall level of CpG methylation was higher in TM cells across all chromosomes (Figure 7C). Together, the results suggest a global increase in net methylation in this system, being highest in TM cells.

Significantly differentially methylated probes were next assessed by genomic context region based on the EPIC manifest designations for relation to CpG island (Island; Open Sea, North Shelf, North Shore, South Shore, and South Shelf) because DNA methylation

levels are known to depend upon genomic context [75]. The percentage of significantly hypermethylated probes was similar for all PGRMC1-HA-mutation states (WT, DM, TM), which differed from MP (Figure 7D, left). By contrast, there were marked differences in the distribution of hypomethylated probes due to PGRMC1-HA mutation status, with DM and especially TM cells exhibiting elevated levels of hypomethylation at CpG islands and both north and south shore sites (which combined would conventionally be associated with increased gene expression), and reduced levels of hypomethylation (increased methylation) in open sea regions (Figure 7D, right). This is consistent with increased levels of open sea methylation (in excess to island and shore sites) leading to the net increased methylation of TM cells, with a minority of local demethylation sites in the vicinity of CpG islands at activated genes. See also the volcano plot of Figure 7B, TM/WT comparison, where positive fold change (logFC >0) is in excess.

The increased level of hypomethylation at Islands and Shores in TM cells was not detectable between 5' untranslated and 3' untranslated regions of annotated coding genes (Figure S3). To examine the possibility that enhancer region CpG methylation is decreased in TM cells we examined the regulatory classifications determined by the methylation consortium. We found that a proportion of cell-type and promoter-associated enhancers exhibited reduced hypermethylation or increased hypomethylation in the TM/DM comparison (Figure S4). This reduced level of enhancer methylation, normally associated with gene activation, occurred in the background of bulk overall increased CpG methylation in TM cells. Since TM and DM cells differ only in the presence of Y180 of the exogenous PGRMC1 protein, we conclude that Y180 phosphorylation status appears to specifically influence the genomic methylation status of cell-type specific enhancers.

We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) enrichment analysis of the methylomics results, using probes from Island or Shore genomics regions only. For KEGG we separated the data into separate hyper- and hypo-methylated gene sets. The KEGG results are presented in Figure 7D, and full GO and KEGG results are provided as Supplemental Information File 1. Each mutation state induced specific unique suites of detected pathways. Most significantly differential KEGG pathways were detected by all three cell comparisons.

Across all comparisons, the most significant enriched GO terms included some associated with embryology and developmental processes (Table 2, Supplemental Information File 1). KEGG results reflected the activities of signal pathways, cell-extracellular interactions, and cancer biology (Table 2, Supplemental Information File 1). E.g., for the TM/DM cell comparison, which differs in only the presence of phosphate acceptor oxygen of PGRMC1-HA Y180, Reactome pathways enrichment suggested extracellular matrix organization, glycoseaminoglycan synthesis, synaptic protein-protein interactions, and collagen formation. KEGG results indicated that the most hypomethylated pathway in TM cells was PI3K-Akt (path:hsa04151), closely followed by "Focal adhesion" (path:hsa04510) and "Pathways in cancer" (path:hsa05200). The PI3/Akt pathway was also among the top 5 in the hypermethylated data set for that comparison, and was not unique to the TM/DM comparison, but was significantly affected in all three comparisons (Supplemental Information File 1). In an accompanying manuscript we predicted reduced PI3K/Akt activity in TM cells on the basis of proteomics pathways enrichment, where the pathway was also significantly differential for all cell comparisons, and we demonstrated reduced phosphorylation of Akt substrates in TM cells [47].

By way of illustrative example, we further explored the biology underyling the pathways analysis by considering the unique KEGG pathways identified for both hyper- and hypo-methylated genes from Figure 7D. 13 hyper-methylated and 7 hypomethylated pathways were unique to the TM/DM comparison (Figure 7D). Their identities are given in Table 3. Hypermethylated pathways included processes related to proteolysis and bacterial infection as well as DNA base excision repair and mismatch repair involved in averting mutations. The latter is striking because we also detected protein abundances involved in pathways associated with DNA damage (p53 effectors, and ATM/ATR), to be reduced in TM cells by proteomics (Figure 4). That led us to examine genomic fidelity to reveal an actual elevated mutation rate in TM cells (Figure 5), which is fully consistent with the methylomics pathways biology. These circumstances suggest strongly that the study reflects true biology related to PGRMC1 Y180 function.

Whereas NNMT was more abundant in TM cells (Figure 6B), CpG sites in its gene were hypermethylated (not shown) as were the sites in many coding genes (Figure S3), which should normally repress transcription. Therefore, the elevated levels of NNMT mRNA appear to be induced by a mechanism independent of genomic gene methylation. For instance, histone methylation status, which we have not assayed, could be influential in determining gene expression levels, or a hypomethylated enhancer may activate transcription despite CpG methylation of the immediate gene locus. Approximately 80% of the genes encoding the 243 differential proteomics heat map of the accompanying paper [47] were significantly differentially methylated between one of the comparisons (not shown), suggesting that epigenetics may contribute greatly to the phenotypes observed for these cell types.

DISCUSSION

We show that PGRMC1 phosphorylation status affects cell metabolism, genomic stability, and epigenetics. Pathway enrichment analysis of proteomics results from an accompanying paper [47] suggested that PGRMC1 phosphorylation potentially played a role in DNA replication, cell cycle, and mitogenic signaling, including the ATM/ATR pathways involved in recognizing genomic damage and inducing repair (Figure 4). Exploratory whole genomic sequencing of those cell lines following thirty passages of cell culture indeed revealed differences in genomic mutations, where the highest rate was observed in the S57A/Y180F/S181A triple mutant, which differs from the S57A/S181A double mutant (DM) by just the phosphoacceptor oxygen atom of Y180. This implies that changes in posttranslational modification of PGRMC1 are involved in the maintenance of genomic integrity. Future research should examine whether this involves previously reported PGRMC1 involvement with G0/G1 checkpoint and/or spindle association [8, 10-14].

We show that perturbation of the regulation of Y180 phosphorylation motif, which is adjacent to S57 in the folded protein [16, 43], can induce considerable changes in cellular plasticity. This may be akin to reprogramming aspects of multicellular regulatory mechanisms back to a state more typical of early embryogenesis. We have demonstrated that dramatic effects occur under these circumstances, but have not demonstrated the mechanisms. Whatever these may be, it is clear that PGRMC1 phosphorylation status is highly relevant to cancer. It may indeed be associated with the altered prognosis of triple negative breast cancers [18]. However our most important finding is that PGRMC1 Y180 affects pathways associated with animal embryology, seemingly by targeting the genomic methylation status of CpG sites in a subset of cell-type and promoter-associated enhancers.

In an accompanying paper we show that Y180 (and Y139) appeared in animal evolution in the common ancestor of Cnidaria and Bilateria [63], concomitantly with the organizer that drives the embryological tissue differentiation processes. PGRMC1 Y180 was therefore evolutionarily acquired before the appearance of chordate organs, and the embryological differentiation processes that give rise to them. Accordingly, these processes could require Y180 phosphorylation, which immediately suggests how PGRMC1 may affect cancer cell plasticity. The increase of hypermethylated CpG sites observed in TM cells (Figure 7, Figure S1) is indeed reminiscent of the characteristic increase in hypermethylation associated with both hESCs and induced pluripotential stem cells (iPSCs). The genome subsequently becomes increasingly hypomethylated during embryogenesis and somatic tissue differentiation [76]. It is as if differentiation processes depended upon the acquisition of Y180 and the organizer of the Cnidaria/Bilateria common ancestor [63], and, upon disturbance with Y180 functions in TM cells, the genomic CpG methylation pattern reverts to a phenotype reminiscent of the undifferentiated state of that ancestral evolutionary stage, prior to the appearance of deuterostome and chordate tissues and body plan.

The activity of nicotinamide N-methyltransferase (NNMT) has been shown to regulate pluripotency in naïve hESCs, where it was proposed to deplete cellular SAM levels and pleiotropically lead to reduced histone methylation, including some genes in the Wnt pathway whose concomitant elevated expression affected pluripotency [62]. PGRMC1 involvement in hESC pluripotency [56] suggests that it could be mechanistically involved in the epigenetic modulation that regulates hESC pluripotency [62]. Perhaps the most striking result we observed was that each PGRMC1 phosphorylation status mutant condition elicited a distinctly unique pattern of genomic methylation (Figure 7). This is a

result we could expect if PGRMC1 played a central role(s) in regulating genomic methylation, with each phosphorylated isoform capable of dynamically engendering different end-points.

The mechanisms of higher animal embryological genomic methylation are set in motion by the organizer, and therefore these processes must represent evolutionary novel metazoan functions. The correlation of appearance of the Y139/Y180 combination and organizer function suggests that these are related to those functions. This new hypothesized PGRMC1 functionality seems to be superimposed upon more ancient PGRMC1 functions, such as sterol synthesis and steroid responsiveness. In support of this hypothesis, all of our mutants responded similarly to cyP450-requiring P4-dependent resistance to doxorubicin-induced death, as well as to AG-205 toxicity (Figure 3).

PGRMC1 is required to maintain the pluripotency of embryonic stem cells by influencing the p53 and Wnt/β-catenin pathways [56]. In an accompanying paper [47] we show that TM cells exhibit lower levels of PI3K/Akt activity, and lowered levels of GSK3-β phosphorylation on an Akt target site. The canonical Wnt pathway is activated when β-catenin translocates to the nucleus to act as a transcription factor after activation by Wnt ligands. Active GSK3-β phosphorylates β-catenin, targeting it for polyubiquitination and proteasomal degradation [55], and Akt phosphorylation of GSK3-β inhibits GSK3-β activity [55]. While our cells are not embryonic, lack of Y180 phosphorylation and low PI3K/Akt activity could correlate with increased GSK3-β activity, and lower β-catenin levels which would attenuate Wnt signaling via TCF/LEF. This suggests that PGRMC1 alterations in the phosphorylation status of Y180, evolutionarily acquired coincidentally with the organizer [63, 64], may be associated with negative regulation of the Wnt pathway by negatively regulating the GSK3-β-dependent destruction of β-catenin.

PGRMC1 has been associated with negative regulation of TCF/LEF in hGL5 granulosa/luteal cells [52] and hESCs [56]. Further experiments are required to explore this concept further in this and other cell models, which should be prioritized.

The propensity for animals and their cells to age was correlated with the loss of pluripotential stem cells in the adult, which was evolutionarily necessary to establish larger collections of clonally communal but functionally specialized cells with diverse differentiation states [77]. That entire biology was associated with the evolution of the tissue and cell-type heterogeneity of bilateral animals, which started with the evolutionary appearance of the organizer [77]. The involvement of PGRMC1/PGRMC2 phosphorylation in cell cycle control mechanisms [10, 11, 14, 78] may accordingly be related to an overarching function associated with the reduction of the immortality potential of primitive eukaryotes, and the necessary evolution of a systematics of differentiation status and replicative control in bilateral animals [77]. This hypothesis merits future examination.

The NNMT pathway has been linked to lifespan and aging [79-82], to sirtuin protein stabilization in positive a feedback loop to the pathway in Figure 6C involved in energy regulation and obesity [83-85], and to epigenetics in cancer and stem cell status [62, 86-88] (reviewed by [89]). To our knowledge we show for the first time that PGRMC1 Y180 modulates this system. TM cells, which differ from DM only by the presence of the oxygen phosphate acceptor of Y180, exhibited the highest level of NNMT, which was accompanied by elevated 1-MNA, the product of NNMT. However, contrary to the expectation if NNMT levels pleiotropically regulated SAM levels and CpG methylation, as reported for histone methylation in hESCs [62], SAM levels were not detectably altered by NNMT depletion in these cells. Nonetheless, irrespective of SAM levels, PGRMC1

phosphorylation status mutants imposed quite specific signature alterations in genomic CpG methylation. TM cells, which expressed the highest NNMT protein levels, exhibited the highest levels of overall genomic methylation (Figure 7C).

In mining our proteomics data for enzymes related to the NAD/NNMT/1-MNA pathway (Figure 6C), we noted that nicotinamide phosphoribosyltransferase (NAMPT) was one of the relatively few proteins less abundant in TM compared to both DM and WT (Figure S2). NAMPT catalyzes a competing reaction to NNMT, whereby nicotinamide is converted to nicotinamide mononucleotide, which is then transformed into NAD^+ by nicotinamide/nicotinic acid mononucleotide adenyltransferase. NAMPT is the rate-limiting enzyme in this pathway, and regulates sirtuin activity by modulating NAD^+ levels [90]. This entire pathway is related to aging processes [91], which were acquired by bilaterian animals along with tissue differentiation and cell cycle control mechanisms to evade cancer [77]. Indeed, these are the very types of biology with which PGRMC1 function is here implicated: suggesting that PGRMC1 phosphorylation could be involved in the mechanism(s) of animal aging. Considering the long co-evolutionary history of Bilateria and PGRMC1 tyrosine phosphorylation sites, this merits future study.

Our results imply that the ability to interchange between phosphorylated states at T178, Y180, S181 and S190 [43] will be critical in supplying correct PGRMC1 function at appropriate times for the cell cycle and state of cell differentiation during early embryology. Clearly, Y180 is an ancient residue that is important for PI3K/Akt activity, a major pathway associated with aging [92].

S181 has been discussed in the literature as a consensus CK2 site [8]. It must be noted in this context that PGRMC1 phosphorylation at S181 was unaffected by knockout of CK2

kinase activity in C2C12 mouse myoblast cells [93], indicating that at least in those cells CK2 was not responsible for S181 phosphorylation. Willibald et al. [94] recently used an antibody against PGRMC1 phospho-S181 (pS181) to argue that S181 phosphorylation is essential in PGRMC1 activation, as well as discussing the presence of phosphorylated PGRMC1 in immunohistochemistry of tissue biopsies [32]. A caveat to these results is that the peptide epitope used to generate the antibody (which was designed and commissioned by M.A.C. while at ProteoSys AG, [18, 95]), corresponded to a CK2 consensus site. CK2 is a promiscuous kinase which gives rise to 10% - 20% of the human phosphoproteome [96]. On Western blots of breast cancer whole cell proteins, that antibody generated a low background smear of signals across many molecular weights, in addition to recognizing exogenously expressed PGRMC1 but not a S181A mutant (not shown). More rigorous characterization of the anti-pS181 antibody specificity would be required to exclude that the immunohistochemistry of tissue biopsies was not detecting increased levels of non-PGRMC1 CK2 phosphorylation sites.

Conclusions

This work suggests that PGRMC1 has been a key yet hitherto unrecognized protein involved in the early evolution of animals, and that its phosphorylation status is capable of influencing biological processes which can destabilize the ordered harmony of the metazoan multicellular collection of functionally specialized cells, with imperatively relevant connotations for human disease. Our work was inspired by the discovery that PGRMC1 phosphorylation differs between breast cancer subtypes differing in estrogen receptor expression, that also exhibit starkly contrasting levels of patient survival [18]. It is wholly feasible that characterization of the signal network surrounding PGRMC1 could lead to novel treatments for cancer, and other diseases involving PGRMC1 [8]. That aspiration could have been greatly advantaged (there has been more than a decade hiatus

in experimental results publication due to lack of resources) if the Australian national competitive grants system had supported this obviously innovative, highly significant, and demonstrably valid [16, 18, 97] work after M.A.C.'s relocation to Australia in 2008.

MATERIALS AND METHODS

Establishment of cell lines and cell culture

Construction of cell lines and culture conditions is described in the accompanying publication [47]. Intracellular redox state measurement by fluorescent redox sensor NpFR1 was performed by flow cytometry as described for NpFR2 [47].

Genomic DNA sequencing

Genomic DNA for sequencing was isolated from one subline of each cell condition. Passaging was repeated every 4 days for thirty passages, with media changes every 48 hours. To extract genomic DNA after thirty passages, approximately $5x10^6$ cells were centrifuged for 5 min at 300 x g. The cells were suspended in 200µL phosphate buffer saline. The purification of total DNA from cells was performed using the DNeasy Blood & Tissue Kit (Qiagen, C.N 69504) following the manufacturer's protocol. The concentration of the dsDNA was measured using a Qubit dsDNA Hs Assay Kit (Life Technologies, Ref Q32851). The samples were sent to Kinghorn Centre for Clinical Genomics (Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst, NSW 2010 Australia) for whole genomic sequencing. DNA for methylation was extracted the same way except we used passage 10 cell lines, consisting of triplicate cultures of MP cells, and respective lines 1-3 of each PGRMC1-HA mutant condition (WT1, WT2, WT3, DM1, DM2, DM3, TM1, TM2, TM3).

MTT cell viability assays for effects of P4 on the dox and AG-205 responses

To assay for proliferative and protective effects of P4, 10^4 cells were seeded per 96 well plate well. For each cell type, n=6 replicates were allowed to adhere for 3 hours after

which viable adherent cells were quantified by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Previous experiments had shown adherence to be complete after 1.5hr (data not shown). Identical replicates were incubated overnight in complete DMEM. After 23 hours the media was exchanged for complete DMEM containing either 1μM P4 or DMSO vehicle control. After a further 1 hour incubation (t0) the media was exchanged for media containing either 1μM P4 or DMSO vehicle control (to assay for proliferative effects, Figure 2A), or with varying concentrations of doxorubicin (dox) (Figure 2B-C) or AG-205, +/- P4 [38]. Cells were incubated for a further 24hr followed by viable cell quantification by MTT assay. The media was discarded, cells were washed with warm PBS and incubated with 100μL of 0.5 mg/mL MTT (Sigma-Aldrich M2128) in phenol red free media (Sigma-Aldrich D1145). After 3 hours incubation at 37°C and 5% CO_2, the media was removed and 100μL of DMSO (EMD Millipore, 317275) was added to each well to solubilize the Formazan crystals. The cells were incubated for 1 hour followed by mixing, and absorbance was read at 570nm using a plate reader (Bio-Strategy P/L, Campbellfield, Vic., Australia). The percentage of viable cells was estimated by normalizing the absorbance of the treated or untreated cells to the average values from panel A.

NNMT shRNA attenuation

Anti NNMT shRNA lentiviral production was as described using mission TRC2-pLKO-Puro series Lentiplasmid (SHCLND, Sigma-Aldrich) scramble shRNA (scr-sh) [47] and shRNA targeting NNMT (Sigma-Aldrich TRCN0000294436, TGCAGAAAGCCAGATTCTTAA). WT and TM cell lines were seeded in 24 well plates until they reached approximately 50% confluency followed by transduction with shRNA virus particles and selection as described [47].

RT-PCR

RNA was extracted by total RNA mini kit (Bio-Rad, 7326820) according to the manufacturer's instructions. cDNA was synthesized in C1000 Touch™ Thermal Cycler using cDNA synthesis kit (Bio-Rad, 1708890) following Bio-Rad's recommended protocol. Real-time qPCR was performed on a CFX96 Touch™ Real-Time PCR Detection System utilizing iTaq™ Universal SYBR® Green Supermix (Bio-Rad, 1725120). NNMT Primers for qPCR were synthesized at Monash University and the sequences were obtained from [98]. NNMT primers used in this experiment were Forward sequence 5'-GAATCAGGCTTCACCTCCAA-3' and Reverse sequence 5'-CCCAGGAGATTATGAAACACC-3'. Actin was used as an internal control Forward sequence 5'GACGACATGGAGAAAATCTG-3' and Reverse sequence 5'ATGATCTGGGTCATCTTCTC-3'.

Western Blot

Cells were lysed with radioimmunoprecipitation assay buffer **(**RIPA buffer) (Sigma Aldrich, R0278) supplemented with protease and phosphatase inhibitor cocktail (Thermoscientific, 88668). After scraping the cells, they were centrifuged at 8000 rpm for 20 minutes (Hermle Centrifuge Z233 M-2) at 4°C. 20µg protein of thirty passage number of MP, WT, DM, and TM were loaded into the wells of a 12.5% SDS-PAGE gel and set to run at 150V for 45 min on Power Pac. Transferring the protein from the gel to a PVDF membrane occurred under wet transfer with 1x Towbin buffer at 40V for 3 hours in an ice bath. The membranes were incubated with 1:2000 diluted Beta-Actin (Sigma Aldrich, AS441) and 1:2000 HA-Tag (Sigma Aldrich, H3663) primary antibodies in blocking buffer overnight at 4ºC with shaking. Next day, the membranes were washed twice with 0.05% PBS-T and incubated with anti-mouse secondary antibody; horse radish peroxidase (HRP), for 1 hour at room temperature. Next, the membranes were washed twice with 0.05% PBS-T and once with PBS. Colorimetric detection of the bands occurred using tetramethylbenzidine as described [47].

Reverse phase protein array

Methods for reverse phase protein arrays are described in the accompanying paper [47]. The following primary antibodies (provider and reagent number, dilution) were used here: Erk1/2-P-Thr202/Tyr204 (CST 4370, 1:100), MEK1/2-P-Ser217/Ser221 (CST 9154, 1:100), MKK4(SEK1)-P-Ser257/Thr261 (CST 9156, 1:100), Rb (CST 9309, 1:200), Rb-P-Ser807/Ser811 (CST 8516, 1:100).

Hyperspectral fluorescence microscopy

UV and visible continuous wave epifluorescence microscopy with multiple excitation wavelength ranges from 335 nm to 532nm and measuring emission in three emission wavelength ranges 450+/- 30 nm, 587+/- 17.5 nm and 700 nm long pass were used. Excitation wavelengths are supplied by low cost multi-LED light source optical fibre coupled to the Olympus IX71 microscope. An Andor/Oxford Instruments iXon 885 Electron Multiplying Charged Coupled Device (EMCCD) was used to capture images typically using an EM gain sufficient to lift the low light auto-fluorescence signal above the 17 electrons per pixel per second readout noise but with minimal contribution from clock induced charge. Gain linearity is ensured by using the Real Gain^{TM} technology (Andor/Oxford Instruments). The camera is operated below -70°C such that thermal noise is negligible. Quantum efficiency of the sensor varies from 50-65% across the range of interest from 450-670nm and the intensity is digitized into ~16K values. Background signal is subtracted from all images which is kept minimal through the use of low fluorescence petri dishes (CELLview, Greiner Bio-One). A single wavelength image may take between 1-5 seconds depending on the sample and wavelength but 1-2 minutes is typically required for the entire stack of images for all spectral channels, , where the features for Figure 1A were as follows.

Feature 1: 'Ratio of Channel 4 to Channel 10',

Feature 2: 'Ratio of Channel 9 to Channel 16',

Feature 3: 'Ratio Channel 2 to Channel 16',

Feature 4: 'Ratio of Channel 5 to Channel 14',

Feature 5: 'Ratio of Channel 2 to Channel 7',

Feature 6: 'Ratio of Channel 9 to Channel 18',

Feature 7: 'Ratio of Channel 7 to Channel 15',

Feature 8: 'Ratio of Channel 7 to Channel 9',

A description of the channels used and their spectral ranges is given in Table 1. During growth under standard mid-log phase growth conditions, hyperspectral imaging was performed on ~300 live cells per cell condition (MP, WT, DM, TM) and the mean cellular intensity in each channel was calculated. Pixel correlations between spectral channels were removed using principal component analysis (PCA), followed by a discriminatory projection to maximally separate the four cell groups (Figure 1A). Technical details of this approach are described by Gosnell et al. [68].

Metabolite preparation

Scr-WT, scr-TM, shRNA NNMT-WT, and shRNA NNMT-TM cells were seeded in Greiner Bio-One Tissue Culture Petri Dish 100mm x 20mm (Interpath, 664160). The cells were rinsed with pre-warmed deionised water with brief shaking. The plates were placed on liquid nitrogen for 15 seconds. Cells were extracted with 1 mL of ice-cold extraction solvent (2:2:1methanol-ethanol-water) and harvested with scraper. The contents were transferred to 1.5 mL Eppendorf tube. The tubes were centrifuged at 4°C for 5 minutes at 16,100 x g and supernatant were transferred to a new 1.5 mL Eppendorf tube. The supernatant was filtered and evaporated dry under a gentle stream of nitrogen gas. The residual precipitates were resuspended in 500 µL of 60% acetonitrile/water (ACN/H_2O) containing m-tyrosine (10 µg/mL) (Sigma, Castle Hill, NSW) as an internal standard.

Metabolite quantification

Metabolomic analysis was performed using an Agilent 1290 Infinity HPLC system equipped with a quaternary pump, degasser, temperature-controlled column and sample compartment coupled to an Agilent 6470 triple quadrupole (QQQ) mass spectrometer with a Double Jet Stream electrospray ionization (ESI) source (Agilent Technologies, Australia).

The column temperature was maintained at 35°C and the autosampler temperature was set at 4 °C. The electrospray (ESI) source settings were as follows: nebulizer gas, 45 psig; drying gas flow rate, 8.0 L/min; drying gas temperature, 300°C; sheath gas temperature, 350°C, sheath gas flow, 10 L/min; capillary voltage, 4000 V; and nozzle voltage, 0 V. The data were acquired positive ionization mode.

Methylnicotinamide samples and samples from Figure 6A were analyzed with a Kinetex HILIC column (50 mm × 2.1 mm, 2.6 µm particle size, pore size of 100 Å, Phenomenex, CA, USA). The mobile phases were 100% HPLC-grade acetonitrile (A) and 10 mM ammonium acetate in HPLC-grade water adjusted to pH 3 with formic acid (B) at a flow rate of 200 µL/min. The solvent gradient began with 95% ACN for 0.5 minutes, decreasing linearly to 35% ACN over 12 minutes. The gradient was maintained at 35 % ACN for 0.5 minutes and increased to 95 % ACN over 0.1 minutes. The mobile phase composition was then held at 95% CAN for 10 minutes to re-equilibrate the column before the next injection. The total time for the gradient program was 24 minutes.

1-MNA was tentatively identified with *m/z* of 137.0633 Da, and an MS/MS score of 99.74. Samples of authentic 1-methylnicotinamide chloride (1-MNA) (CAS Number 1005-24-9, Sigma-Aldrich # M4627), 2-methylnicotinamide (2-MNA) (CAS Number 58539-65-4, Ark Pharm Arlington Heights, IL 60004 , USA, #AK-39636) and N-methylnicotinamide (N-MNA) (CAS Number 114-33-0, Sigma-Aldrich #M4502) were

injected at three concentrations (1, 10 and 100 µg /mL) to confirm identification of 1-MNA. The compound was unambiguously identified as 1-MNA with retention time (RT) of 7.33 min and *m/z* of 137.0717, and not 2-MNA (RT 0.96 min, *m/z* 137.0719) or N-MNA (RT 0.97 min, *m/z* 137.0709).

Cellular extracts except Figure 6A were analyzed with a HILIC-Z column (50 mm × 2.1 mm, 2.6 µm particle size, 100 Å pore size, Agilent Technologies, CA, USA) equipped with a guard column of the same stationary phase. The mobile phases were HPLC-grade acetonitrile:water (95:5) (A) and water (B), both containing 20 mM ammonium formate and 0.1% formic acid (pH ~3) at a flow rate of 400 µL/min. The solvent gradient started at 100% A and was held for 0.5 minutes, followed by a linear gradient from 100% A to 60% A over 9 minutes. The gradient was maintained at 60% A for 0.5 minutes and returned to 100% A over 0.1 minute. The solvent was then held at 100% A for 5.4 minutes to re-equilibrate the column before the next injection. Injection volume was 2 µL. The total time for the gradient program was 15 minutes.

Analysis of the set of samples was preceded and followed by injection of five concentrations of 1-MNA (10 µg/mL to 1 ng/mL in log steps) and six concentrations of S-adenosyl methionine (SAM) (100 µg/mL to 1 ng/mL) (Sigma-Aldrich, Castle Hill, NSW). In addition, after every 15 samples a blank injection and a mixture of 1-MNA, m-tyrosine and SAM was run to verify that there was no carryover between samples and that retention time and detector sensitivity had not changed during the course of running the samples.

The selective detection and quantitation of 1-MNA was achieved by monitoring the transition of *m/z* 137 → 108 using a fragmentor voltage of 120 V and collision energy of 20 V, with the further requirement that retention time matched that of the authentic compound (ca. 2.7 min). m-tyrosine was quantitated by monitoring the transition *m/z* 182 –> 136 (fragmentor voltage 135 V, collision energy 20 V, RT = 4.9 min) and SAM was

quantitated by monitoring the transition *m/z* 399 -> 136 (fragmentor voltage 100 V, collision energy 20 V, RT = 9.1 min). The fragmentor voltage and collision energy values were determined by optimizing system response to authentic 1-MNA, m-tyrosine and SAM. Limits of quantitation were determined by regressing the log(detector response) to log(concentration) response over the range of serial dilutions for each standard, and by observing where the dose/response curve departed from linearity.

Effect of 1-MNA on morphology

MiaPaCa-2 cells expressing DM PGRMC1-HA were seeded at 25% confluency and cultured overnight. The media was removed and the cells were incubated in the presence of the indicated amount of 1-MNA (Sigma-Aldrich, Castle Hill, NSW) for 24 hours. The 1-MNA stock was made in sterile H_2O. Five random images were taken per treated culture and assigned random numbers 1-20. These were given to a blinded scorer who decided for each image whether given cells were round or elongated.

Methylome Assay

Genomic DNA from each cell subline were processed using the Illumina Infinium HD Methylation Assay which interrogates >850,000 CpG sites. The four cell lines were processed in triplicate equating to 12 samples, being three replicates of MP cells, and independent cell lines 1-3 for each of the PGRMC1 states [47]. The array was prepared at the Australian Genome Research Facility (AGRF) following the manufacturer's instructions. Quality checking of the samples was performed by Nanodrop Spectrophotometer and resolution on a 0.8% agarose gel at 130 V for 60 minutes. 500ng total DNA was bisulfite converted with Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA), following the manufacturer's standard protocol. Amplified DNA samples were fragmented and resuspended DNA was loaded onto a BeadChip. The BeadChip was incubated overnight while DNA fragments anneal. After hybridization the array was imaged on the Illumina iScan system.

Methylation data were processed using in R version 3.5.1 (www.r-project.org). Data were assessed using the hg19 build of the human referene genome annotated in the IlluminaHumanMethylationEPICanno.ilm10b4.hg19 bioconductor annotation. Quality control of the array was assessed with the R package Lumi [99]. Probes on the sex chromosomes, cross-reactive probes and probes with known SNPs at the CpG site were excluded. Following filtering 812,817 probes remained. The cleaned data were normalized with the SWAN normalization, and sample relationships examined. Genome-wide differential methylation analysis was undertaken using Limma [100]. Multiple testing correction was applied using the Benjamini and Hochberg method. Differential methylation in each group was observed on volcano plots and heatmaps.

Due to the excessive quantity of differentially methylated probes with small effects we refined the data used for functional analysis to the top 2000 most variable probes prior to pathways analysis. These probes explained >90% of the total variance among the first two principle components while preserving a reasonable number of intragenic probes. Pathways enrichment was undertaken in R using the *enrichPathway* function in the ReactomePA package [101], which implements a one-tailed hypergeometric test for overrepresentation. Multiple testing adjustment was applied with the Bonferroni & Hochberg FDR. KEGG pathways analysis was undertaken using the *kegga* function in the R package in LIMMA. Enrichment was calculated for genes from the top 2000 most variable probes with a p-value <0.05. The gene sets were split to those with hypomethylated probes (logFC<0) and those with hypermethylated probes (logFC>0) for enrichment. Enrichment of Gene Ontology (GO) terms was undertaken using the GOstats package [102]. Ontology assignments are calculated using a hypergeometric test for overrepresentation on the significantly differentially methylated probes (p<0.05) among the 2000 most variable. Multiple testing adjustments were applied using the FDR.

Intersects of common pathways from KEGG and Reactome analyses were assessed using the UpSetR package [103].

LIST OF ABBREVIATIONS

1-MNA: 1-methylnicotinamide

adjP: Benjamini-Hochberg adjusted *p*-value

AG-205: AG-205 PGRMC1 inhibitor

CK2: casein kinase 2

cyP450: cytochrome P450

CYP51A: lanosterol 14-alpha demethylase

cytb5: cytochrome b_5

Dap1: damage-associated protein 1

DM: PGRMC1-HA S57A/S181A double mutant

Dox: doxorubicin

GLP-1: glucagon-like peptide-1

GO: Gene Ontology

GSK3-β: Glycogen synthase kinase 3-β

HA: hemagglutinin

hESCs: human embryonic stem cells

KEGG: Kyoto Encyclopedia of Genes and Genomes

LC/MS: Liquid chromatography/mass spectrometry

LDL: low density lipoprotein

LDLR: low density lipoprotein receptor

MAPR: membrane-associated progesterone receptor

MP: MIA PaCa-2 cells

mPRα: membrane progestin receptor α

NAMPT: nicotinamide phosphoribosyltransferase

NNMT: nicotinamide-N-methyltransferase

NpFR1: Naphthalimide-flavin redox sensor 1

NpFR2: Naphthalimide-flavin redox sensor 2

P4: Progesterone

PGRMC1: Progesterone Receptor Membrane Components 1

PGRMC1-HA: hemagglutinin-tagged PGRMC1

RPPA: reverse phase protein array

SAM: S-adenosylmethionine

SH2: Src homology 2

SH3: Src homology 3

TCF/LEF: T-cell factor/lymphoid enhancer factor

TM: PGRMC1-HA S57A/Y180F/S181A triple mutant

WT: PGRMC1-HA wild-type

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Methylomics data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-8218. Genomic sequencing results are available as NCBI Bioproject Accession: PRJNA400337, SRA Run Selector SRP118430 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA400337).

Further data are available in a supplemental information file available from the journal web page.

Competing interests

M.A.C. is scientific advisor to and minor shareholder of Cognition Therapeutics, a company developing sigma-2 receptor ligands against Alzheimer's disease. This work was performed independently of and without input from the company. The authors declare that they have no other competing interests.

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Authors' contributions

Conceptualization, M.A.C.; Methodology, M.A.C., P.P.A, B.M.T., E.M.G., M.G., E.J.N, L.A.W., P.A.W, and M.P; Formal Analysis, B.T.M., P.P.A., S.D.P., R.H.T., P.A.W., M.L., M.G., L.G., T.F., T.F., M.P. and M.A.C.; Investigation, B.M.T., P.P.A., S.L.T., J.F., P.A.W., L.A.W., S.G. and M.W.; Resources, M.P., E.J.N., H.N., T.F., E.M.G, and L.A.W.; Writing – Original Draft, M.A.C., B.M.T.; Writing – Review & Editing, M.A.C., B.M.T., P.P.A., M.P., E.M.G; Supervision, M.A.C, J.A.J., and E.J.N.; Project

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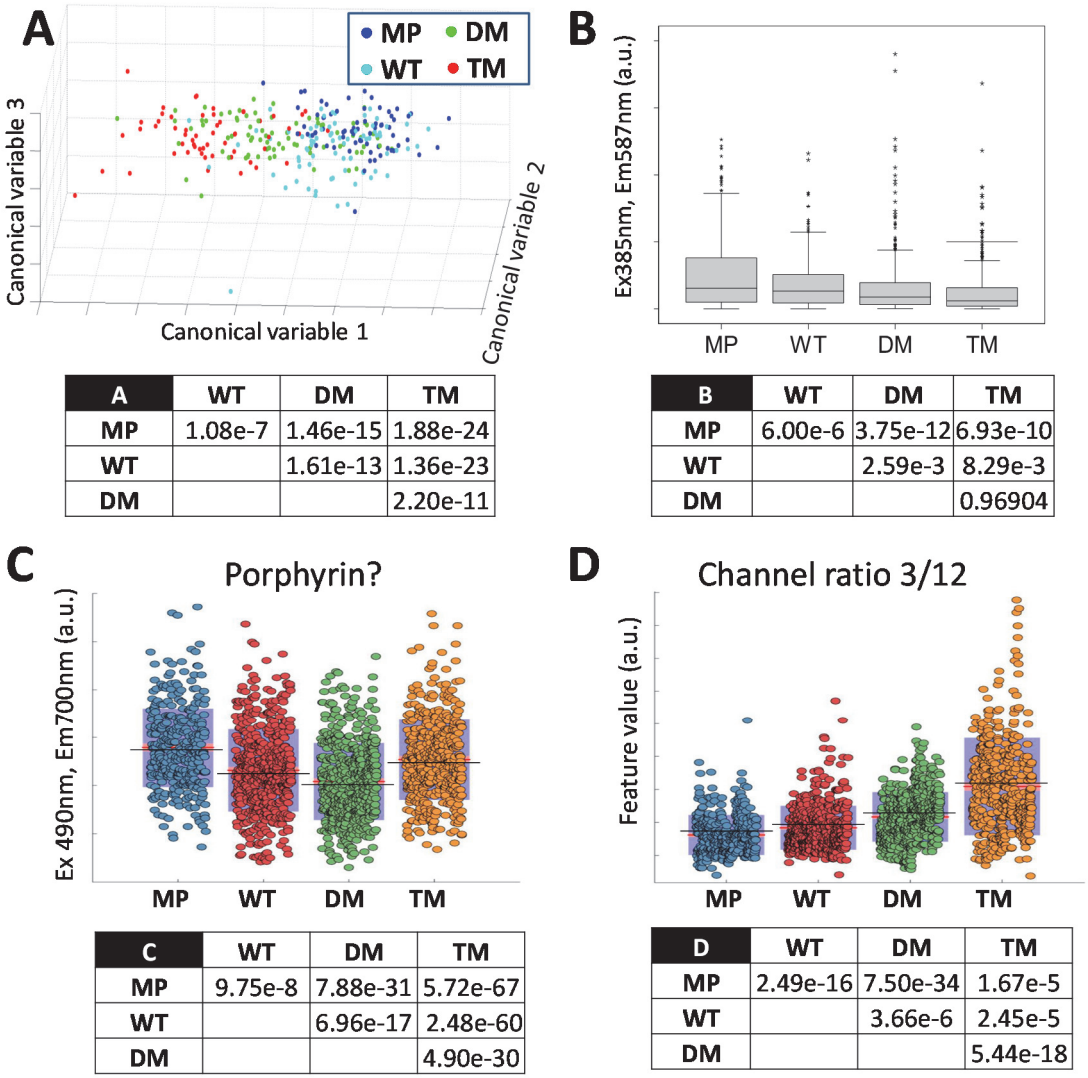
FIGURES & LEGENDS



A	WT	DM	TM
MP	1.08e-7	1.46e-15	1.88e-24
WT		1.61e-13	1.36e-23
DM			2.20e-11

B	WT	DM	TM
MP	6.00e-6	3.75e-12	6.93e-10
WT		2.59e-3	8.29e-3
DM			0.96904

C	WT	DM	TM
MP	9.75e-8	7.88e-31	5.72e-67
WT		6.96e-17	2.48e-60
DM			4.90e-30

D	WT	DM	TM
MP	2.49e-16	7.50e-34	1.67e-5
WT		3.66e-6	2.45e-5
DM			5.44e-18

Figure 1. Hyperspectral autofluorescence reveals metabolic differences between cells expressing different mutant PGRMC1-HA proteins.

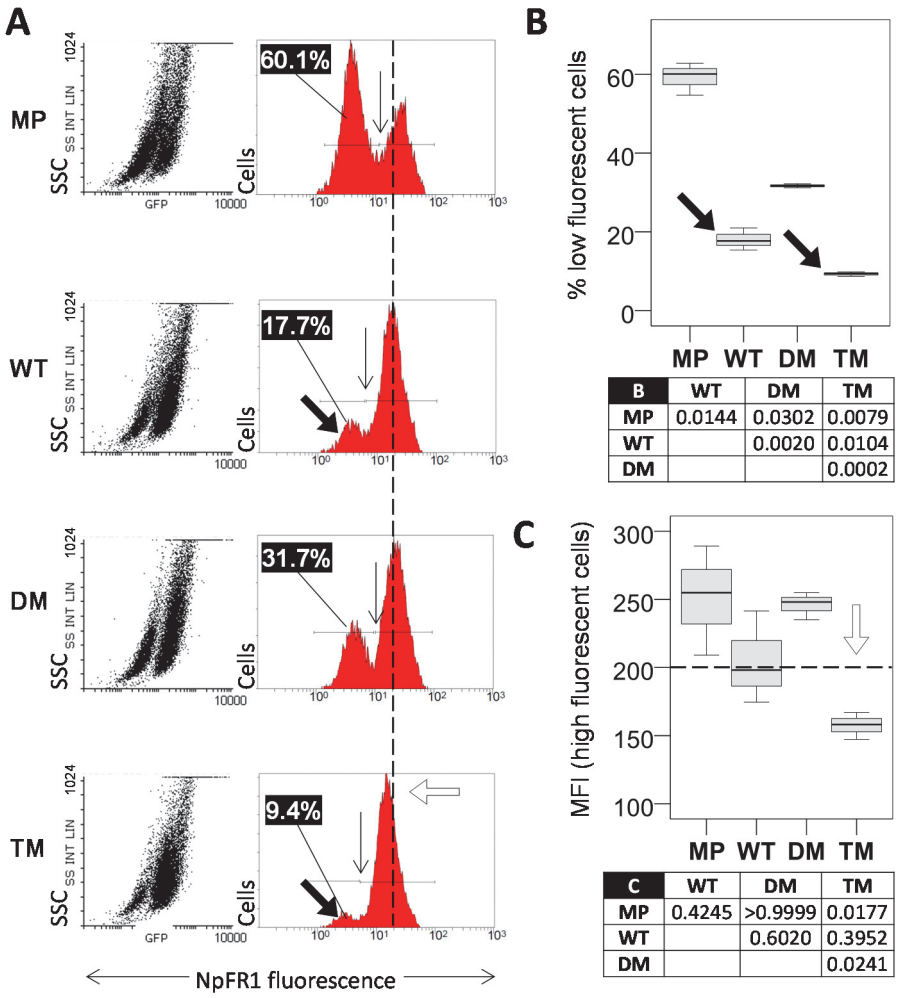
(A) Principal component analysis (PCA) followed by linear discriminant analysis (LDA) reveal hyperspectral autofluorescence parameters that significantly discriminate between cell types. The panel provides a three dimensional depiction of the three most significant canonical variables revealed by PCA and LDA as described [68] that differ according to PGRMC1-HA phosphorylation status. In order to statistically analyze the cluster separations, an additional LDA projection of cell data was carried out, this time with two classes of cells chosen at a time. Their average spectra were projected onto a common direction identified by this additional LDA. The cell distributions were then

tested by the Kolmogorov-Smirnov test. The table provides *p* values from Kolmogorov-Smirnov test pairwise discrimination analysis. The eight features analyzed are described in the methods.

(B) Mean cellular intensity of hyperspectral autofluorescence channel 3 [375nm(Ex), 450nm(Em)], which corresponds primarily to flavin emission, is significantly affected by PGRMC1-HA phosphorylation status. Boxplots were generated in SPSS. The table shows Kruskal-Wallis test *p* values for the pair-wise condition comparisons performed on primary emission data.

(C) Mean cellular intensity of hyperspectral autofluorescence channel 18 [495nm(Ex), 700nm(Em)], which may reflect porphyrin or protein-bound red-shifted flavin emission [70], is significantly affected by PGRMC1-HA phosphorylation status. The table provides Kolmogorov-Smirnov test *p* values from pair wise comparisons [68].

(D) The ratio of hyperspectral autofluorescence channels 3 [375nm(Ex), 450nm(Em)] to channel 12 [435nm(Ex), 587nm(Em)] differs significantly between cells. The table follows C.



B	WT	DM	TM
MP	0.0144	0.0302	0.0079
WT		0.0020	0.0104
DM			0.0002

C	WT	DM	TM
MP	0.4245	>0.9999	0.0177
WT		0.6020	0.3952
DM			0.0241

Figure 2. NpFR1 reveals differences in cytoplasmic redox status.

(A) NpFR1 flow cytometry results. Scatter plots to the left depict green fluorescence on the x axis, and side scatter on the y axis. In the right panels the y axis represents cell number. The numbers at the upper left of each right panel are the fraction of the population in the respective left hand low fluorescent peak. Vertical arrows show the boundary between cell populations, and the inset numbers in the left of each panel represent the percentage of cells in the less oxidized left hand cell population. The vertical dotted reference line represents the 200 fluorescence units, and the open arrow highlights TM fluorescence. We have repeated this experiment on numerous occasions. The actual ratio of low to high abundance distribution for a cell type tends to vary between experiments, however the relative proportions of cells in the low fluorescent population between cell types remains relatively constant (MP > DM > WT > TM).

(B) Boxplots showing mean and sample distribution for % cells in the less oxidized population of replicates of (A).Sample size is n=3, being one each of independent cell lines 1-3 for WT, DM and TM, and triplicates of the MP cell line. The table shows ANOVA post-hoc Dunnet's T3 *p* values for the indicated pairwise comparisons.

(C) Boxplot of median fluorescent intensity (MFI) of the oxidized cell populations from (A). The horizontal dotted reference line and open arrow are identical to (A). The table shows ANOVA post-hoc Bonferroni *p* values for the indicated pairwise comparisons.

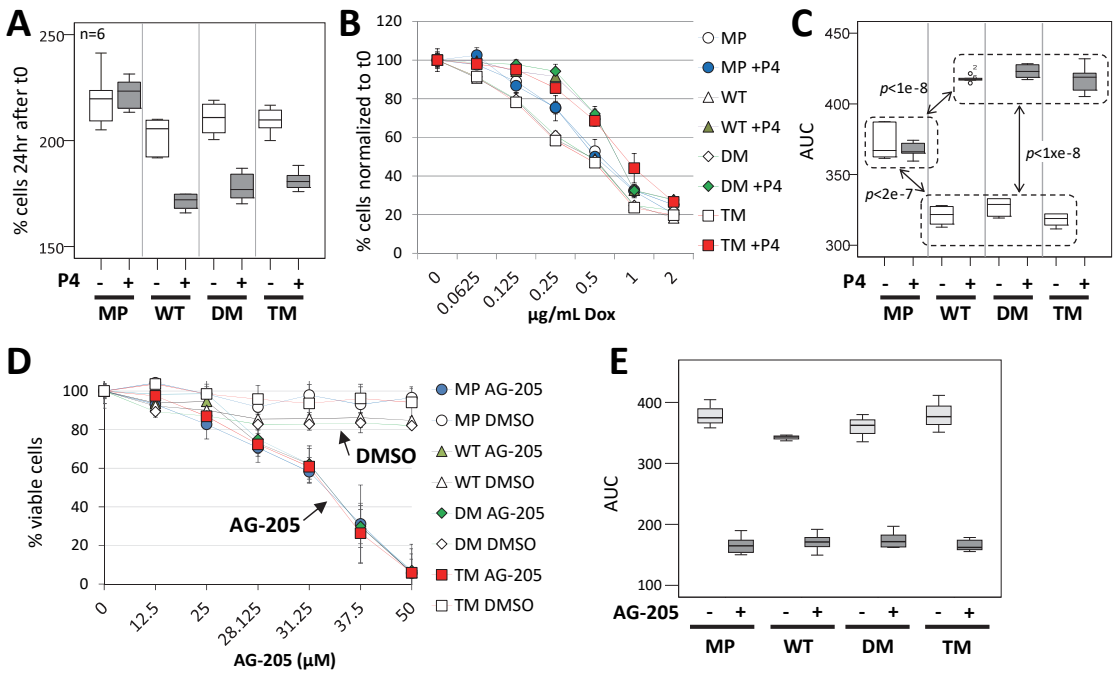


Figure 3. PGRMC1 phosphorylation status does not affect P4-dependent resistance to doxorubicin toxicity or resistance to AG-205-induced cell death.

(A) P4 reduces cell proliferation of cells expressing all PGRMC1-HA proteins. The panel shows boxplots of viable cells for n=6 replicates. The viability of cells pretreated with P4 (dark boxes) or DMSO vehicle control (light boxes) after 23 hr as described in methods were allowed to grow a further 24 hours and the level of MTT formazan was quantified as a proxy for viable cell numbers. "% cells after 48 hr" is presented relative to the signal obtained after adherence for 3 hr. No significant differences were observed between non-P4 treated cell pairings, except MP v. TM (p=0.021, 2 way ANOVA). Pair wise comparisons of P4-treated MP vs any of P4-treated WT, DM or TM, or of +/-P4 treatment for WT, DM and TM, were significantly different at the p<0.00001 level (2 way ANOVA). MP cells +/- P4 were not different (p=0.55, two way ANOVA). Considering only P4-treated cells, MP differed in P4 response from all other cell types ($p<1x10^{-10}$), and WT differed marginally from TM (p=0.034) by one way ANOVA and post-hoc Bonferroni test.

(B) P4-protection of MP cells from doxorubicin-induced cell death is facilitated by over-expression of PGRMC1-HA proteins (WT, DM & TM). Cells were grown as in (A), except at t0 doxorubicin (Dox) was added at the indicated concentrations, followed by 24hr incubation. Because of altered cell proliferation during pretreatment with P4 (A), all signals at t0 were expressed as a percentage of the averaged control sample without Dox at t0 to assess the effects of P4. Data points represent the averages ± s.d. of n=6 replicates.

(C) Boxplots of area under the curve (AUC) results for all data points with dox addition from (B). Two way ANOVA dox treatments were statistically significant (F=1292.237, df=1, df2=40, p< 1=e-8), with Partial Eta Squared indicating 97% effect size in the data. Considering pairwise comparisons of cell classes, DM v. TM (p=0.014) and WT v. DM (p=0.05) were significantly different. Pairwise comparison between the AUC levels for +/-P4 for each cell class yielded no difference for MP, but $p<1x10^{-8}$ for other cell types, +/- P4. One way ANOVA post-hoc Bonferroni pairwise comparisons for -P4 cells

revealed that samples within dotted boxes did not differ significantly, whereas all pairwise *p* values were less than those indicated for comparisons between boxes.

(D) AG-205-induced cell death is unaffected by PGRMC1 phosphorylation status. Cells were incubated in the presence of the indicated AG-205 concentrations (n=8: 3x line 1, 3x line 2, 2 x line 3) or DMSO vehicle control (n=3: 1x each cell line), and percentage viable cells was calculated relative to untreated cell controls (n=9: 3 replicates per cell line) using MTT assay.

(E) AUC results for values from D greater than 25μM reveal no significant differences in response to AG-205 treatment between cell lines ($p>0.85$, post-hoc Bonferroni after 1 way ANOVA for AG-205 treatment). The apparently greater survival of DM cells at 25 μM AG-205 observed in this panel was never observed in multiple other repeat experiments.

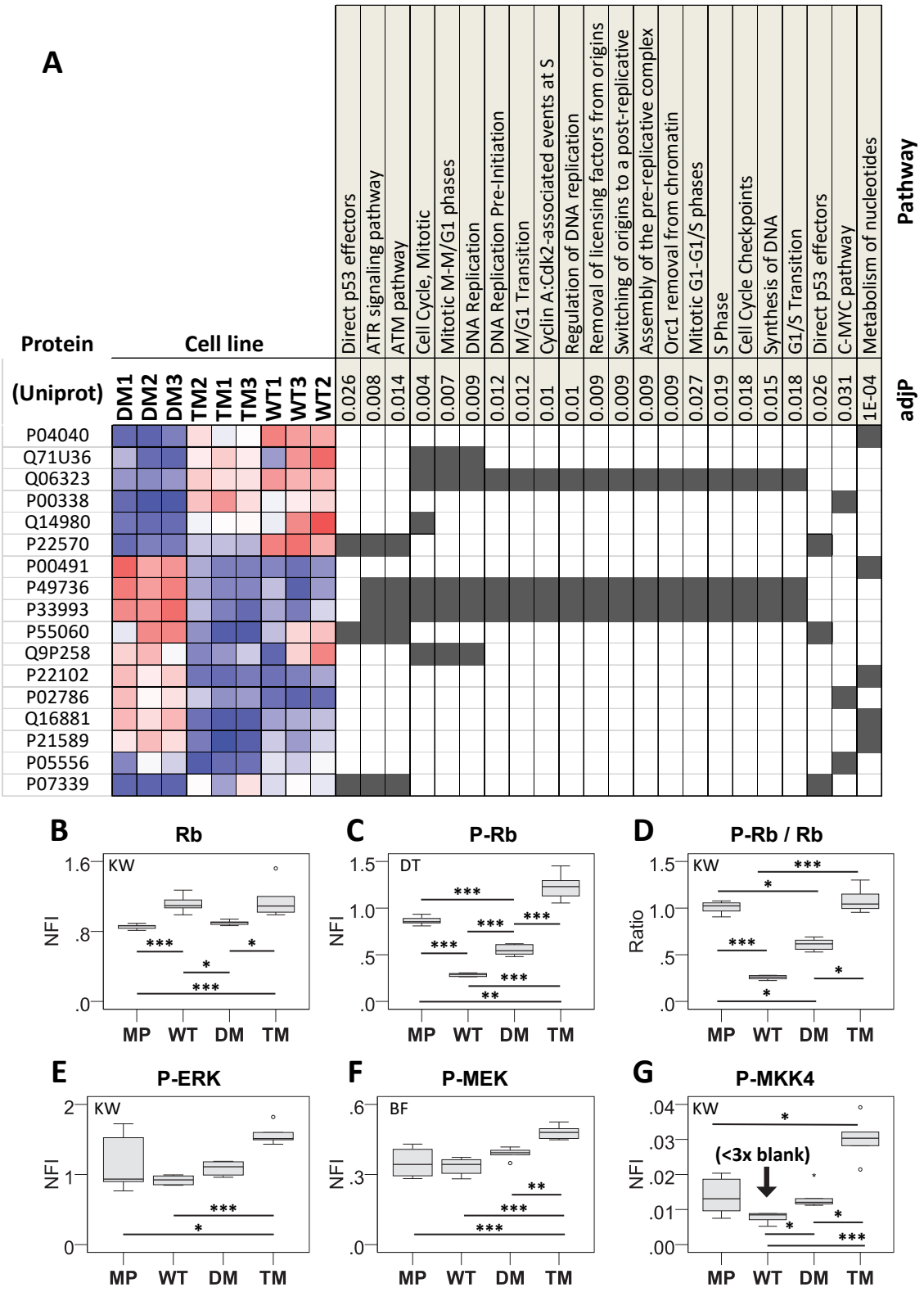


Figure 4. Pathways enrichment suggests altered cell cycle control proteins.

(A) Abundances of proteins detected as significantly differentially abundant in indicated Pathways Commons pathways by enrichment analysis (Accompanying paper [47]) at the adjP < 5% significance level.

(B, C, E-G) Average reverse phase protein array (RPPA) normalized fluorescent intensity (NFI) from the indicated antibodies (described in methods) is plotted from 6

replicate measurements. NFI is normalized to protein content. Significance levels are * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Conventions follow those of the accompanying paper [47].

(D) The ratio of average NFI of the anti-pRB antibody to that of the RB antibody.

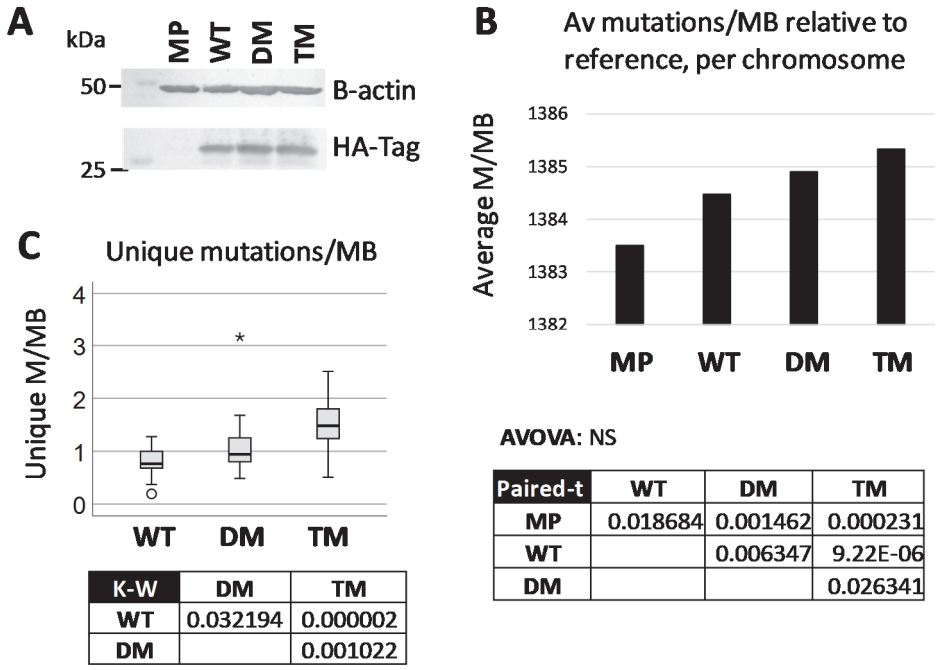


Figure 5. PGRMC1 phosphorylation status effects DNA mutation rate.

(A) Western blot of cells following thirty passages showing expression of HA-tag. Beta actin was used as a control.

(B) Average mutations/MB relative to reference human genome for 23 chromosomes excluding Y. ANOVA gave no significance due to inter-chromosomal variation in mutation rates. However paired t-tests (2 tail) produced the pairwise comparison *p* values in the box.

(C) Unique mutations per cell line relative to human reference genome and other genomes in the analysis. Mutations/MB are plotted for 23 chromosomes excluding Y. The box shows *p* values for Kruskal-Wallis (K-W) post-hoc pairwise comparisons.

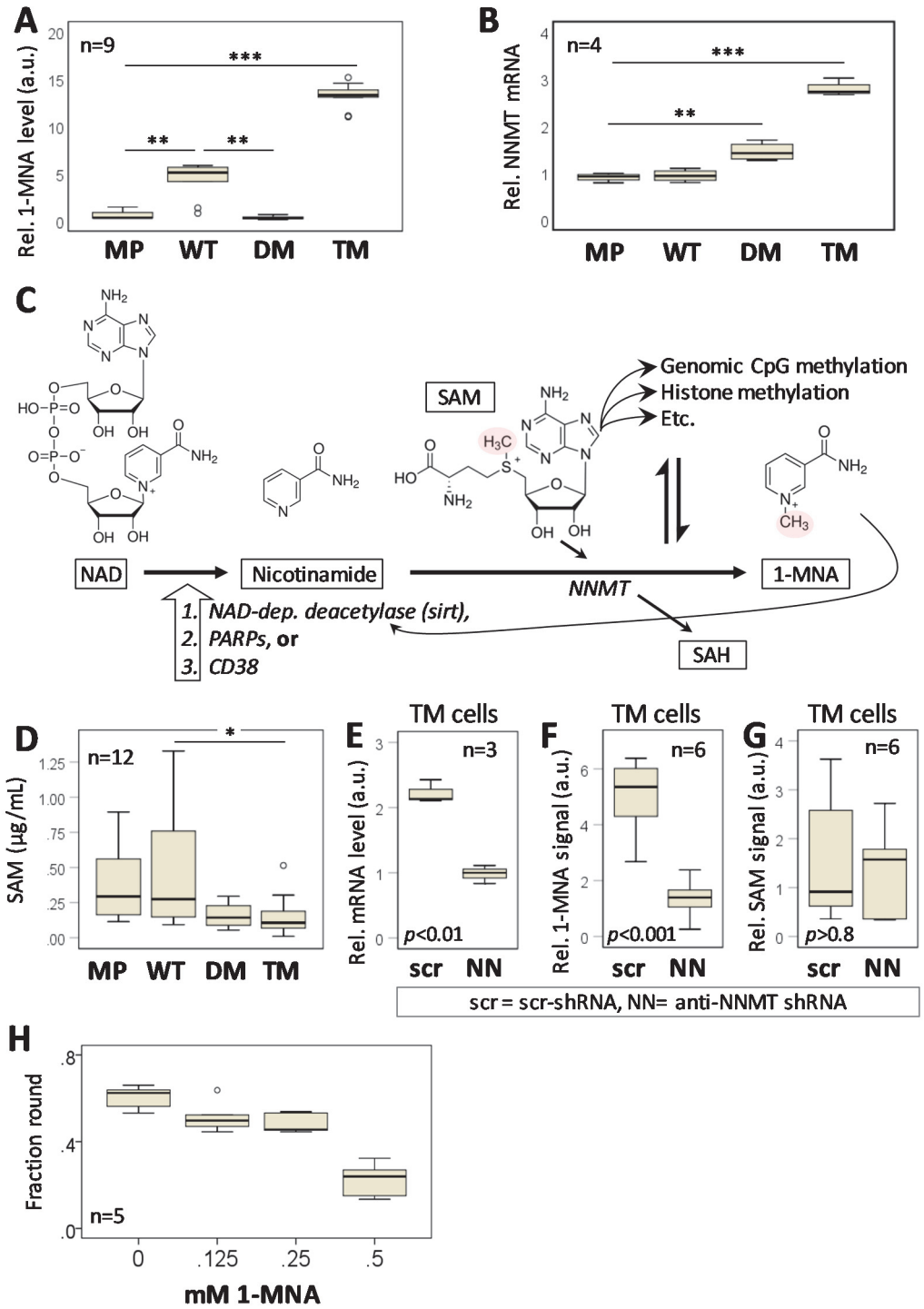


Figure 6. PGRMC1 phosphorylation is associated with NNMT pathway regulation
(A) 1-MNA levels determined by triple quadrupole MS/MS. The y axis represents 1-MNA ion intensity (arbitrary units; a.u.). Samples represent technical triplicates of each of three independently derived stable cell lines or replicates to generate n=9 per condition, or n=3 for MP control. ANOVA post-hoc Tukey's HSD $p<0.01$ (**) or $p<0.001$ (***).

(B) Quantification of NNMT mRNA levels in the four cell types by RT-PCR. Quantification cycle (Cq) results were quantified using the $2^{\Delta\Delta Cq}$ method using beta actin as an internal control. Statistical significance portrayal follows A. (ANOVA post-hoc Tukey's HSD).

(C) Model of the NNMT and 1-methylnicotinamide pathway. Nicotinamide is produced from NAD by NAD-dependent deacetylases (e.g. Sirtuins), PARPS or CD38 [104]. NNMT transfers a methyl group of S-adenosylmethionine (SAM) to nicotinamide to produce 1-methylnicotinamide (1-MNA), producing S-adenosyl homocysteine (SAH). This reaction is in competition with methylation reactions including DNA and histone methylation by SAM [62]. 1-MNA can feed back to stabilize sirtuin 1 [83].

(D) Metabolomics quantification of S-adenosyl-Methionine (SAM) levels for the indicated cell lines, representing n=12 (four technical replicates for each of three independent cell lines per PGRMC1 condition).

(E) RT-PCR quantification of NNMT mRNA levels after treatment by an NNMT-specific shRNA (NN) or a random scramble shRNA control (scr) in TM cells stably expressing lentiplasmid-driven shRNAs. RT-PCR Methods follow A. $p < 0.003$ (2-tailed T test). Methods follow B.

(F) 1-MNA ion intensities in cells from E, determined following methods from A, using two technical replicates of each of three biological replicates per cell condition (n=6). Labels follow E. The result was significantly different by T-test ($p<0.001$) after removal of one Scr outlier (panel displayed) or by Mann-Whitney U test ($p<0.002$) including the outlier.

(G) SAM ion intensities in the cells from E were not significantly different (T-test). Labels follow E.

(H) 1-MNA attenuates rounded morphology in DM cells. Cells were treated with the indicated concentration of 1-MNA and scored as either morphologically round or elongated by a blinded scorer. Indicative images are shown. Results for n=5 for each 1-MNA concentration are shown in the boxplot. 0.5 mM 1-MNA was significantly different ($p<0.0001$, ANOVA, post hoc Tukey HSD) to all other treatments.

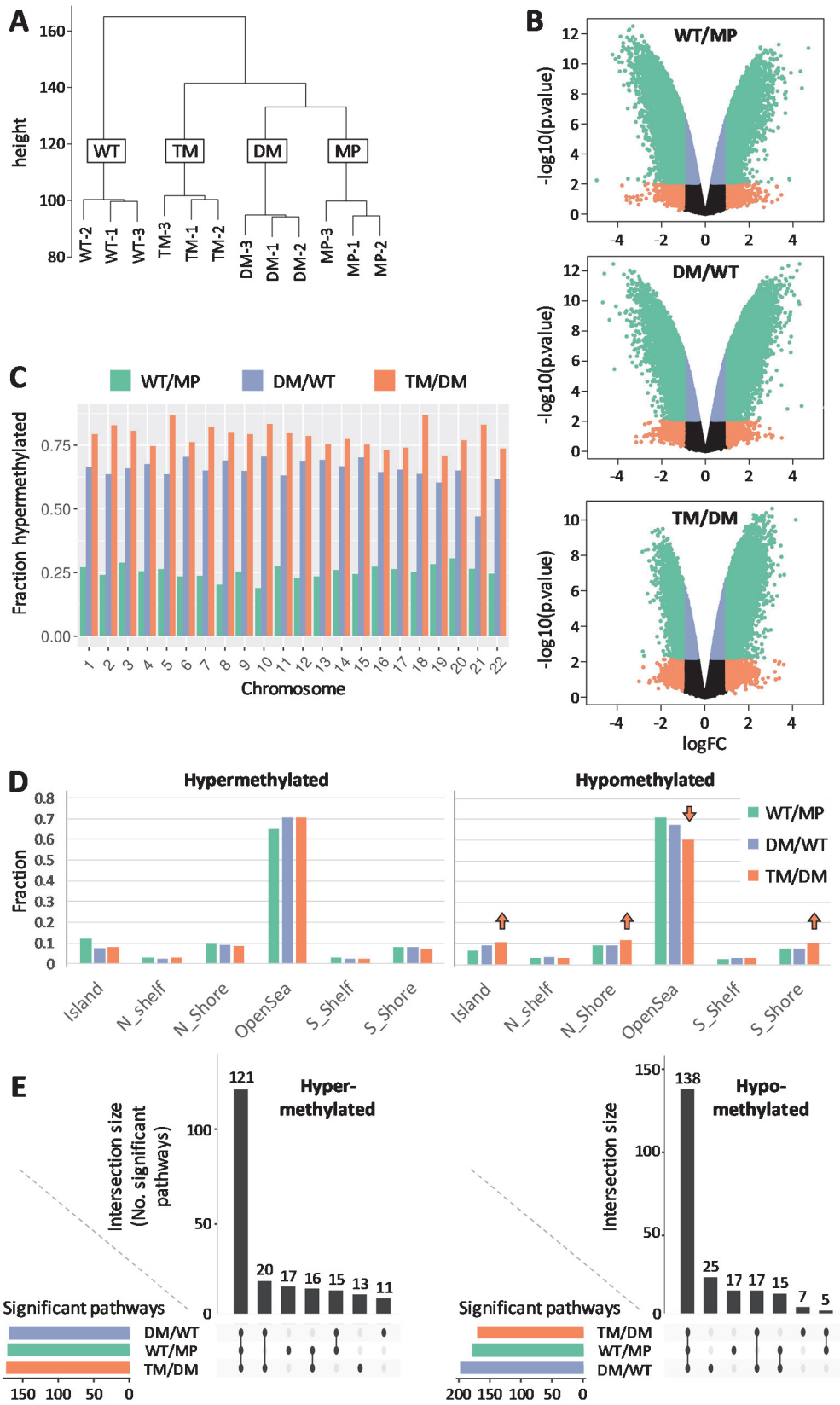


Figure 7. PGRMC1 phosphorylation mutants elicit distinct patterns of genomic methylation

(A) Hierarchical clustering of methylation data from all cell lines sampled in triplicate. Clustering was applied using 520,031 probes with a coefficient of variation > 0.10. Height

is calculated using the UPGMA method in the Lumi package and equates to the average distance between two members of two groups.

(B) Volcano plots of the WT/MP, DM/WT and TM/DM comparisons. The log2 fold change (FC) (x axis) is plotted relative to the log10 *p*-value (y axis) to visualize the magnitude of the most significant features. Probes with an adjusted *p*-value < 0.05 and absolute log FC > 1 are plotted in green. Probes with an absolute logFC > 1 that were not significant are plotted in orange. Probes with a logFC < 1 that were significant at adjusted *p*-value < 0.05 are plotted in blue. Black probes were not significant.

(C) Significant probes in each differential methylation comparison were extracted and the fraction of hypermethylated or hypomethylated probes per chromosome calculated. Methylation levels are higher in TM cells across all chromosomes.

(D) CpG feature classifications for differentially methylated probes. The percentage of all significant probes that were hypermethylated or hypomethylated per CpG feature is presented for each comparison. Up arrows point to relatively greater fractions of Island and Shore genomic regions for the TM/DM comparison. The down arrow points to reduced OpenSea hypomethylation in the TM/DM comparison.

(E) Intersections of significant KEGG pathways between WT/MP, DM/WT and TM/DM. KEGG enrichment was applied separately to significant hypermethylated and hypomethylated probes. The main bar chart presents the number of pathways common to each comparison. Each bar represents an intersect notated by linked by dots on the x-axis. The number of significant pathways per comparison is presented as a horizontal bar chart. The KEGG results are in Supplemental Information File 1 and the list of pathways unique to each comparison are provided. Pathways analysis of probes genes corresponding to the top 2000 most variable differentially methylated probes in CpG Island or Shore chromosomal regions. Probes were separated into hypermethylated and hypomethylated data sets from D, including only probes from Island or Shores. Enriched Gene Ontology (GO) pathways enrichments are shown. The identities of pathways uniquely differential to one of the three cell type comparisons (WT/MP, DM/WT, or TM/DM) for both hypo- and hyper-methylated data sets are available in Supplemental Information File 1, as are corresponding Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

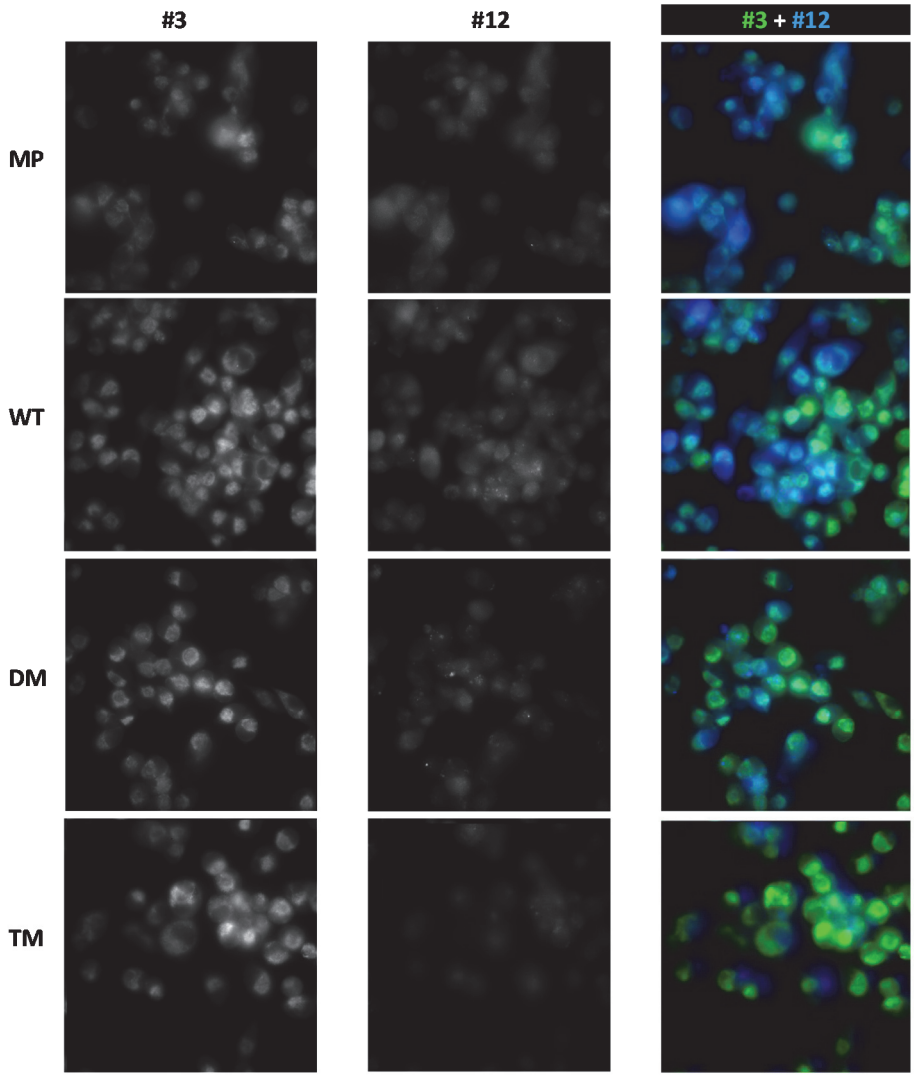


Figure S1. Representative hyperspectral autofluorescence cell images from the measurements of cells from Figure 1D.

Individual channels #3 and #4 as listed in Table 1 (left) and the same two channels superimposed (right).

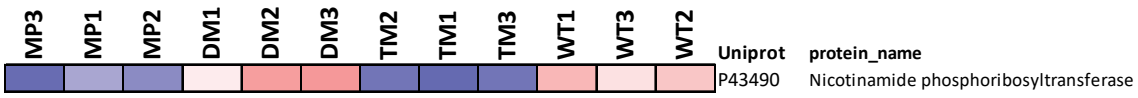


Figure S2. SWATH-MS proteomic quantification of Nicotinamide phosphoribosyltransferase.

The figure shows the abundance profile of P43490 nicotinamide phosphoribosyltransferase from the SWATH-MS proteomics quantification of the accompanying manuscript [47].

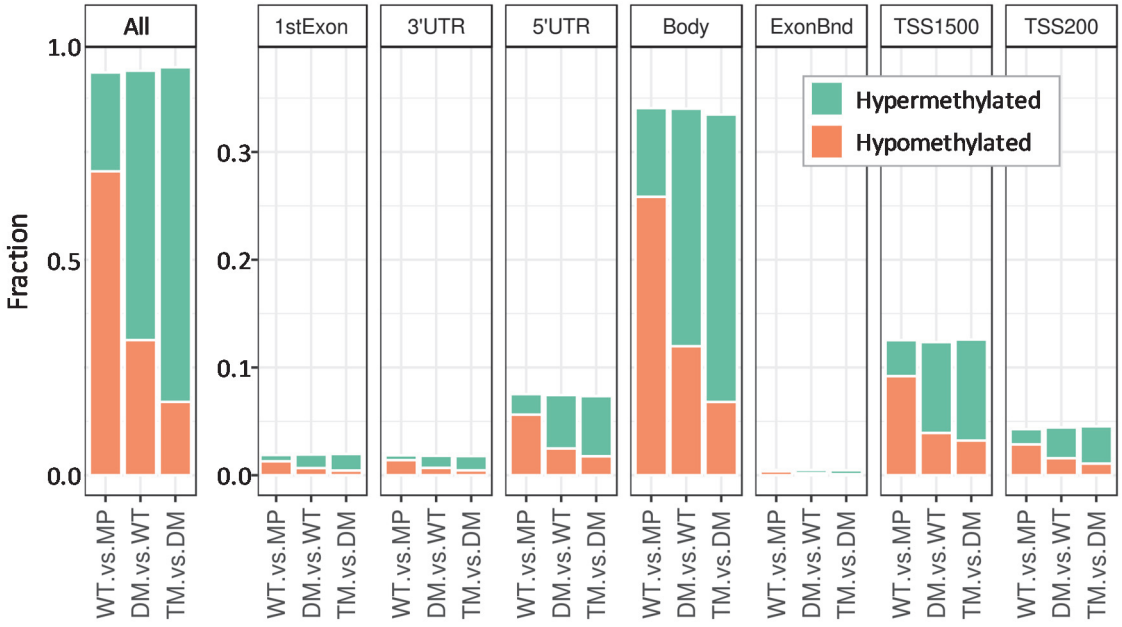


Figure S3. Methylation status of CpG associated with annotated coding genes.

Significant differentially methylated probes were annotated with the UCSC gene feature and the proportion of probes attributed to each feature counted. The y-axis shows the frequency of hypermethylated-hypomethylated probes per feature, and the x-axis shows the difference in those frequencies between WT/MP, DM/WT and TM/DM. 3'UTR: 3' untranslated region; 5'UTR: 5' untranslated region; Body: Between the ATG and stop codon; irrespective of the presence of introns, exons, TSS, or promoters; ExonBnd: exon boundaries: TSS1500/TSS200: within indicated number of residues of transcription start site (TSS).

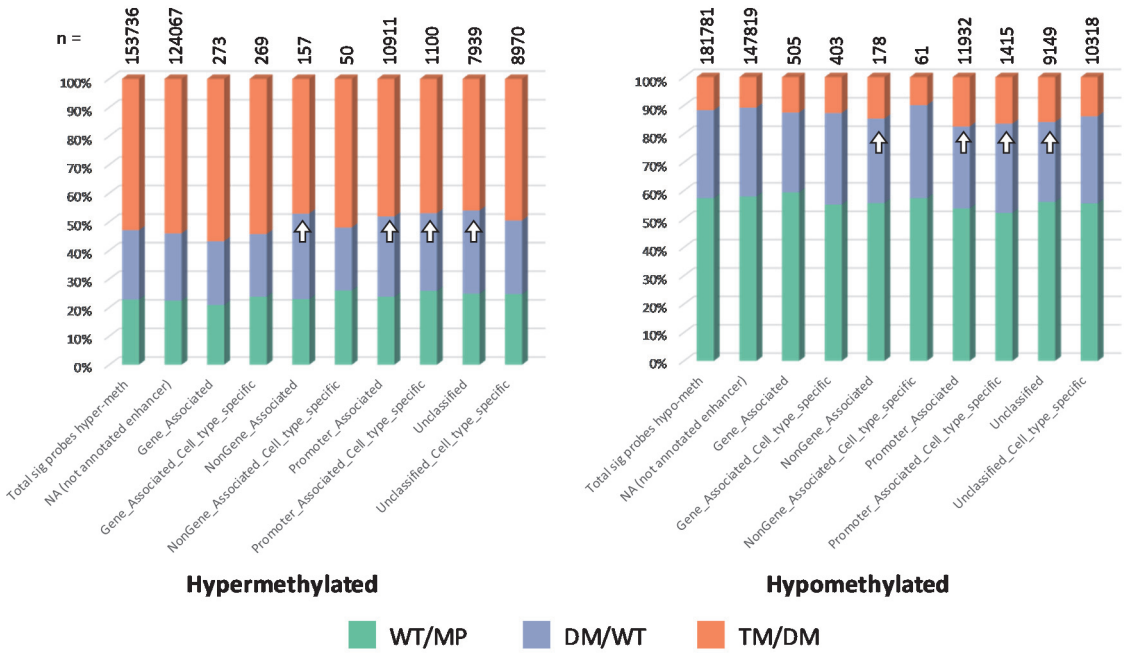


Figure S4. Methylation status of CpG associated with annotated enhancers.

Significant differentially methylated probes were annotated with the 450k_Enhancer feature and the proportion of probes attributed to each feature counted. Arrows indicate increased hypomethylation and reduced hypermethylation of enhancers in the TM/DM comparison.

Channel	Excitation (Ex)/nm +/- 5 nm	Emission (Em)/nm
1	335	450 +/- 30 nm
2	365	450+/- 30 nm
3	375	450+/- 30 nm
4	335	587 +/- 17.5 nm
5	365	587+/- 17.5 nm
6	375	587+/- 17.5 nm
7	385	587+/- 17.5 nm
8	395	587+/- 17.5 nm
9	405	587+/- 17.5 nm
10	415	587+/- 17.5 nm
11	425	587+/- 17.5 nm
12	435	587+/- 17.5 nm
13	455	587+/- 17.5 nm
14	470	587+/- 17.5 nm
15	495	587+/- 17.5 nm
16	405	700 long pass
17	455	700 long pass
18	495	700 long pass

Table 1. Spectral channel filters employed for hyperspectral autofluorescence imaging. For further details of this approach see Gosnell et al. [68].

Comparison	GO_ID	FDR	PValue	sigGO.Term
MP/WT	GO:0032501	8.1E-07	1.85E-10	multicellular organismal process
	GO:0048731	2.03E-06	9.28E-10	system development
	GO:0007275	5.75E-05	4.09E-08	multicellular organism development
	GO:0030154	5.75E-05	6.05E-08	cell differentiation
	GO:0048869	5.75E-05	6.58E-08	cellular developmental process
	GO:0048856	0.00013	1.79E-07	anatomical structure development
	GO:0022610	0.000141	2.26E-07	biological adhesion
	GO:0032502	0.00017	3.52E-07	developmental process
	GO:0023052	0.00017	3.67E-07	signaling
	GO:0009653	0.00017	3.89E-07	anatomical structure morphogenesis
WT/DM	GO:0032501	1.79E-07	4.14E-11	multicellular organismal process
	GO:0009653	2.27E-05	1.05E-08	anatomical structure morphogenesis
	GO:0048731	5.71E-05	4.5E-08	system development
	GO:0050896	5.71E-05	5.28E-08	response to stimulus
	GO:0023052	6.44E-05	7.44E-08	signaling
	GO:0007165	0.000153	2.12E-07	signal transduction
	GO:0007154	0.000227	3.7E-07	cell communication
	GO:0051239	0.000227	4.2E-07	regulation of multicellular organismal process
	GO:0051716	0.000241	5.01E-07	cellular response to stimulus
	GO:0007275	0.000325	7.51E-07	multicellular organism development
TM/DM	GO:0032501	2.71E-07	6.56E-11	multicellular organismal process
	GO:0048731	4.52E-05	2.19E-08	system development
	GO:0009653	5.33E-05	3.91E-08	anatomical structure morphogenesis
	GO:0007275	5.33E-05	5.16E-08	multicellular organism development
	GO:0007399	0.000113	1.37E-07	nervous system development
	GO:0050896	0.000141	2.16E-07	response to stimulus
	GO:0032502	0.000141	2.39E-07	developmental process
	GO:0048856	0.000173	3.36E-07	anatomical structure development
	GO:0030154	0.000214	4.67E-07	cell differentiation
	GO:0048869	0.000365	8.84E-07	cellular developmental process

Table 2. GO pathways enrichment results. The top ten GO enrichments for each cell comparison from Figure 7. Full results are available in Supplemental Information File 1.

Data set	PathwayID	Pathway
Hypermeth	path:hsa04145	Phagosome
	path:hsa03050	Proteasome
	path:hsa05150	Staphylococcus aureus infection
	path:hsa05133	Pertussis
	path:hsa00983	Drug metabolism - other enzymes
	path:hsa05130	Pathogenic Escherichia coli infection
	path:hsa03410	Base excision repair
	path:hsa03430	Mismatch repair
	path:hsa04964	Proximal tubule bicarbonate reclamation
	path:hsa05014	Amyotrophic lateral sclerosis (ALS)
	path:hsa04979	Cholesterol metabolism
	path:hsa04216	Ferroptosis
	path:hsa00051	Fructose and mannose metabolism
Hypometh	path:hsa05203	Viral carcinogenesis
	path:hsa04672	Intestinal immune network for IgA production
	path:hsa04657	IL-17 signaling pathway
	path:hsa00561	Glycerolipid metabolism
	path:hsa04977	Vitamin digestion and absorption
	path:hsa01230	Biosynthesis of amino acids
	path:hsa00920	Sulfur metabolism

Table 3. Unique KEGG pathways detected for TM/DM hyper and hypo-methylated gene sets. The identities of the 11 hypermethylated and 7 hypomethylated pathways unique to the TM/DM comparison from Figure 7E are given. Full results are available in Supplemental Information File 1.

Supporting Information file

Supplemental Information File 1. A zip archive containing excel files with the most significant pathways enrichment results from methylomics analysis including those of Figure 7E, Table 2 and Table 3. The archive unpacks as five separate folders, each containing the following pathways analysis results.

A) All GO enrichments for each cell comparison significant below the adjP =0.001 level.

B) All GO enrichments that were uique to a particular cell comparison.

C) KEGG enriched pathways for each cell comparison, with analyses performed separately on hypermethyalted (files labelled up) and hyperemethylated (files labelled down) probes. These correspond to the pathways of of Figure 7E.

D) All KEGG enriched pathways from C which were unique to a particular cell comparison.

E) All All Reactome enriched pathways for each cell comparison significant below the adjP =0.05 level.