Figure 1

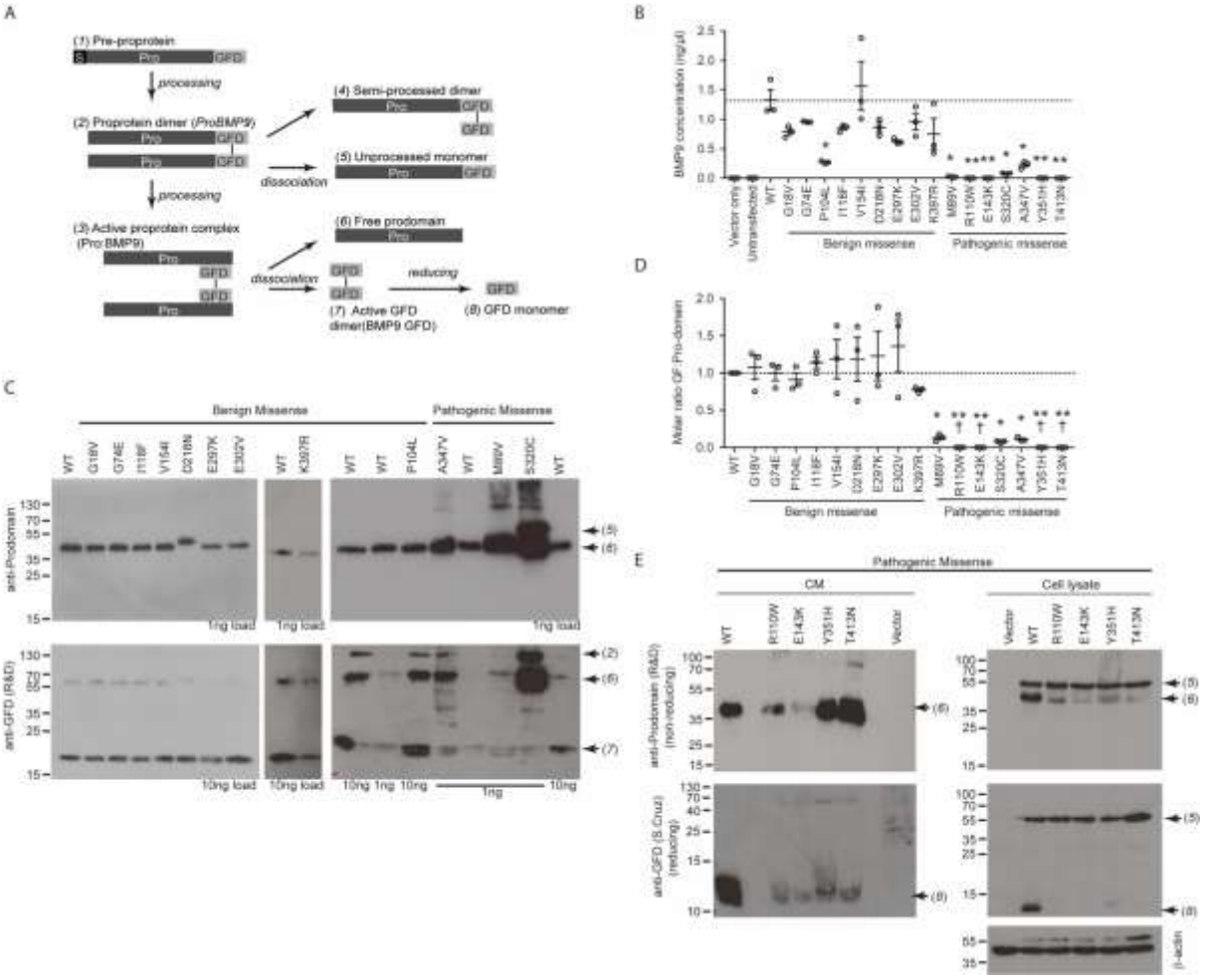

Figure 1: Characterisation of Expressed BMP9 Mutant Proteins. (a) Schematic of different potential species of BMP9. Numbers in brackets correspond to labels on western blots. (b) Conditioned media from HEK-EBNA cells expressing Pro:BMP9-WT or missense pBMP9 variants were serially diluted and assayed for BMP9 GFD levels by ELISA. Data are the mean ± SEM from three independently generated batches of media. Paired Dunnett's multiple comparisons test; **P*<0.05, ***P*<0.01 (c) Western blots of variants in conditioned media in non-reducing conditions. The volume loaded was normalized according to concentration based on ELISA. Blots are representative of n=3 separate expression batches. (d) The ratio of the band intensities of the growth factor and prodomain for each mutant was normalized to WT. † indicates mutants for which GFD concentration was too low to quantify.

Data are the mean ± SEM from three independently generated batches of media. Paired Dunnett's multiple comparisons test; **P*<0.05, ***P*<0.01 (e) Western blotting of those variants not readily detected by ELISA. Cell lysates (30 μg of total protein) and conditioned media (3 μl of Pro:BMP9-WT or 45 μl of Pro:BMP9 mutants and media from the empty vector control) were loaded**.**

Figure 2

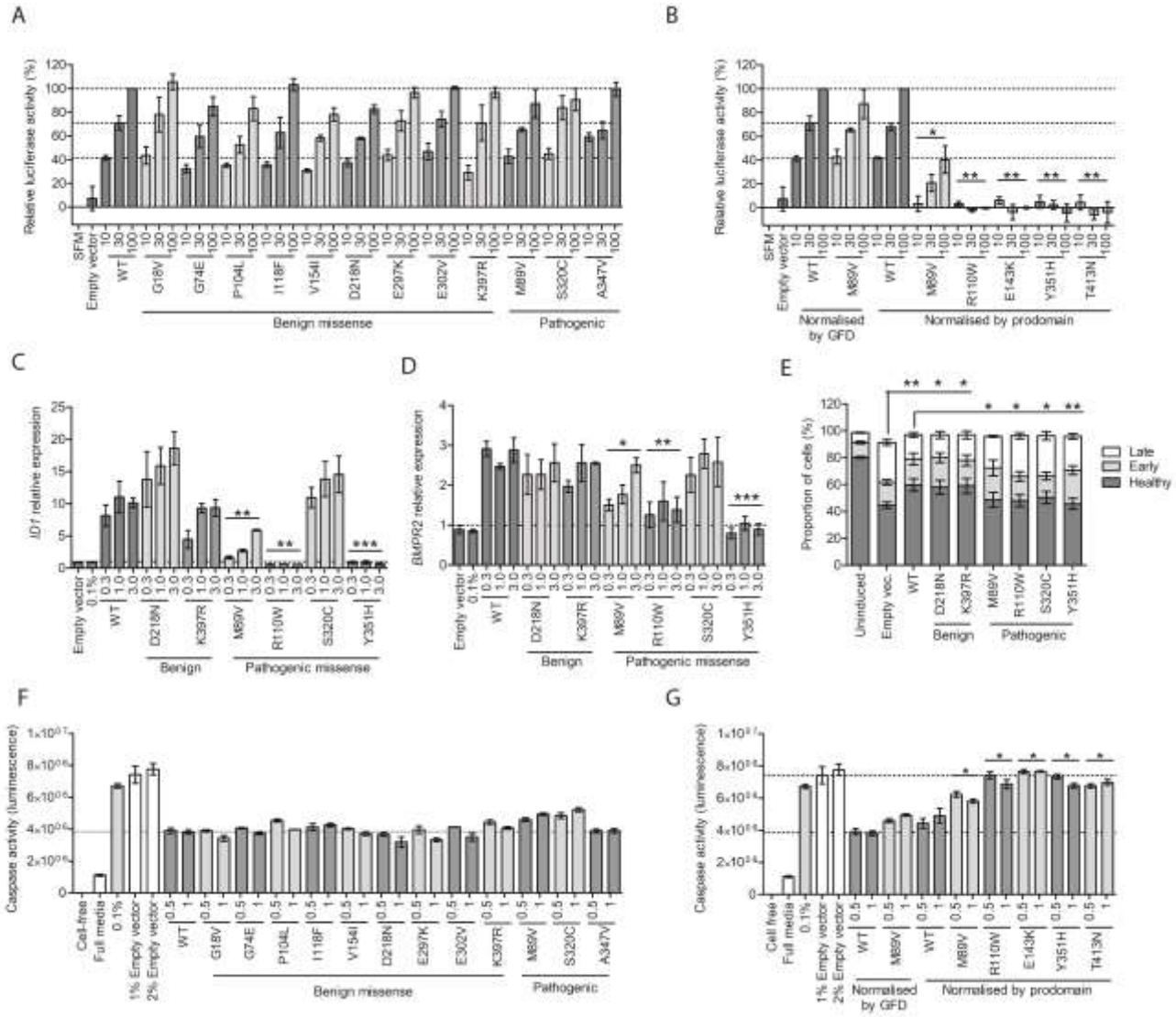

Figure 2: Loss of Activity in Pro:BMP9 Mutants Predicted to be Pathogenic. (a,b) C2C12 cells transfected with human ALK1, BRE-*luciferase* and TK-*Renilla* plasmids were serum-starved followed by treatment with Pro:BMP9 variants (10, 30, 100 pg/ml) for 6h and luciferase assay. The Firefly:*Renilla* luciferase ratios were calculated for each sample and data normalized, with cells treated with 100 pg Pro:BMP9-WT designated as 100%, and SFM-treated cells SFM as 0%. Data are mean ± SEM from three independently generated batches of media. van Elteren test; *P_{adj}<0.05, **P_{adj}<0.01. (c,d) Transcriptional responses of BOECs to Pro:BMP9-WT and variants based on quantification of the prodomains. Cells were starved in EBM2/0.1% FBS and then treated with Pro:BMP9-WT and variants (0.5, 1.5,

5 ng/ml) for 4 h. The expression of (c) *ID1* and (d) *BMPR2* mRNA were normalized to *B2M* and are expressed as fold change relative to EBM2/0.1% FBS. Data are mean ± SEM from three independently generated batches of media. van Elteren test; $^{**}P_{adj}<0.01$ $^{***}P_{adj}<0.001$. (e) BOECs were treated overnight with 5 ng/ml Pro:BMP9-WT or variants (normalized according to the amount of prodomain) in EBM2/2% FBS followed by induction of apoptosis by addition of TNFα and cycloheximide (TNF/CHX) for 3.5 hours. Cells were stained with Annexin V-FITC and propidium iodide prior to flow cytometry analysis. Cells were assigned as healthy (AV^-/PI^-), early apoptotic (AV^+/PI^-) or late apoptotic/necrotic (AV^+/PI^+). Data are the mean ± SEM from three independently generated batches of media. Paired Dunnett's multiple comparisons test; $^{*}P<0.05$. (f,g) PAECs were starved overnight in EBM2/0.1% FBS to induce apoptosis in the presence of Pro:BMP9-WT or variants (0.5, 1 ng/ml). Each BMP9 treatment was supplemented with conditioned media from HEK-EBNA cells transfected with empty vector to equalize the total volume of conditioned media per well (2% v/v for 1 ng/ml, 1% v/v for 0.5 ng/ml). Apoptosis was measured by Caspase-GLO® 3/7 assays. Data are mean ± SEM from ~~for~~ three independent batches of media. van Elteren test; $^{*}P_{adj}<0.05$

Figure 3

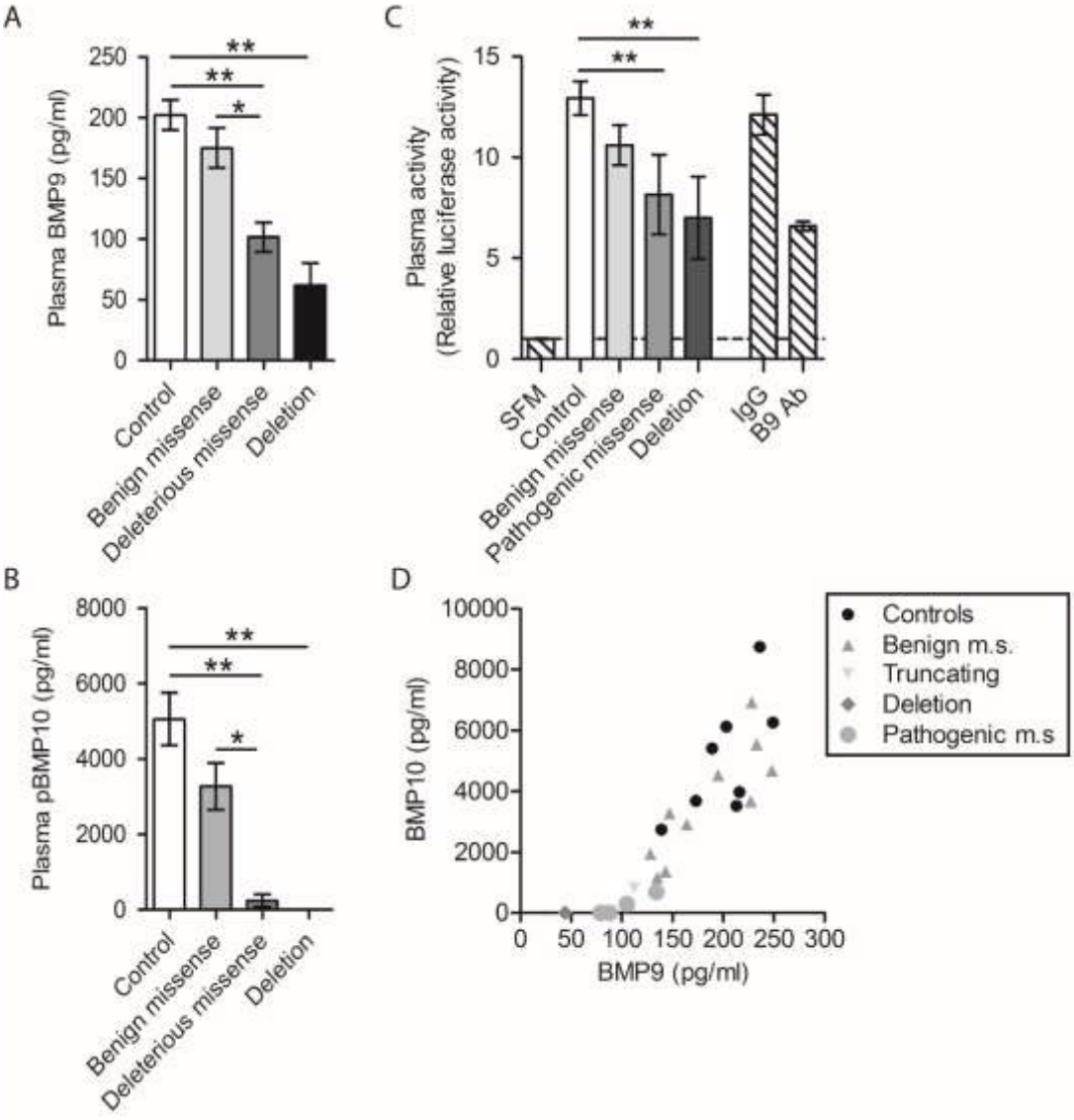

Figure 3: Loss of active BMP9 in PAH patients carrying putatively pathogenic *GDF2* alleles. (a) BMP9 GFD concentration in EDTA-plasma from diseased, *GDF2* heterozygous females and healthy age-matched females was measured by ELISA Data are mean ± SEM; one-way ANOVA; **P*<0.05, ***P*<0.01. (b) HMEC1-BRE cells quiesced in serum free media overnight were treated with 5% plasma for 6 hours before luciferase activity was assessed. Data are mean ± SEM; t-test (c) As for a, but for pBMP10 concentration. (d) The concentration of pBMP10 plotted against the concentration of BMP9 GFD in EDTA-plasma.

Data are mean ± SEM; one-way ANOVA; *P<0.05, **P<0.01. n=8 healthy controls, 11 benign missense, 4 pathogenic missense and 3 deletion (one of which is an early truncation).

Figure 4

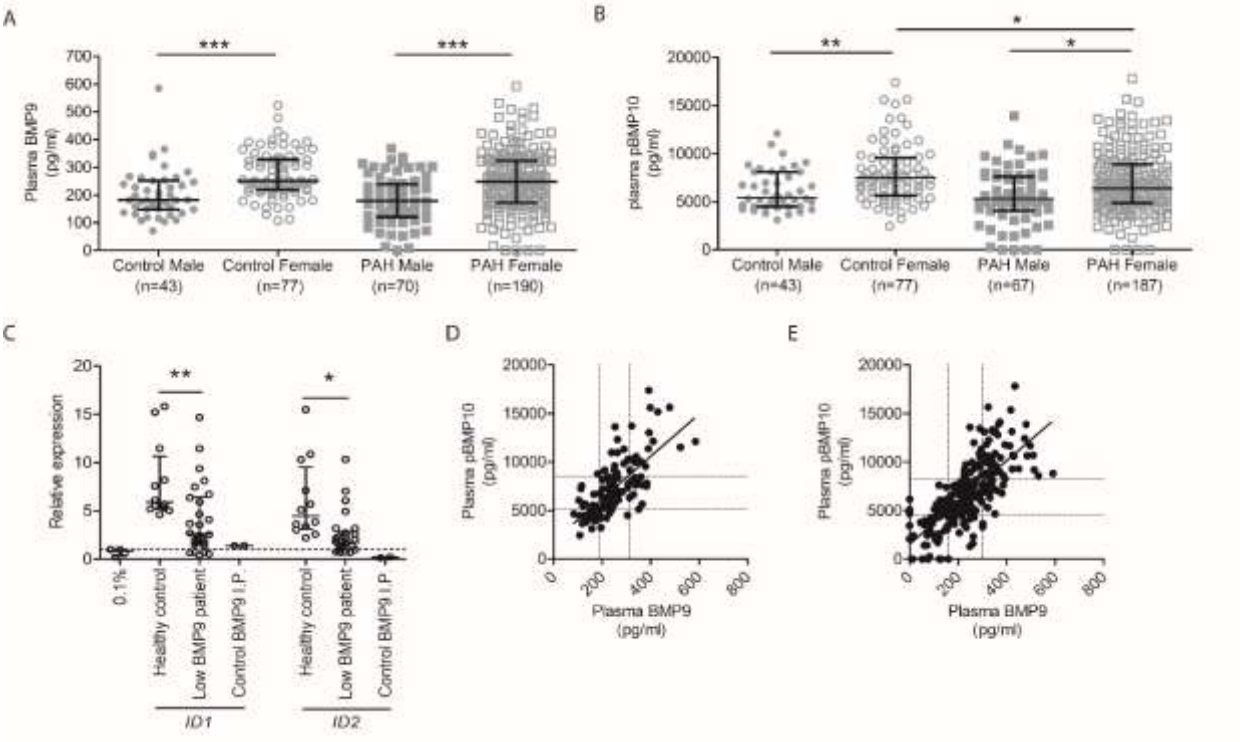

Figure 4: Plasma BMP9 and pBMP10 levels are not reduced in PAH, but a subset of PAH patients exhibit reduced plasma BMP9 and pBMP10 levels. Plasma samples collected from control subjects and PAH patients were assayed for (a) BMP9 and (b) pBMP10. Data are presented as median ± interquartile ranges (Kruskal-Wallis test; ***P*<0.01, ****P*<0.001). (c) hAECs quiesced in 0.1% basal media were incubated with 3% EDTA-plasma for 1 hour. Expression of *ID* mRNA was measured by qPCR and normalized to *B2M* and data expressed as fold-change relative to the EBM2/0.1% FBS control. (mean ± SEM; t-test). (d,e) Plasma BMP9 levels were plotted against plasma pBMP10 levels in (c) control individuals (n=120) and (d) PAH patients (n=187). Dotted lines represent 25% and 75% percentiles. Spearman correlation is shown by a solid regression line.