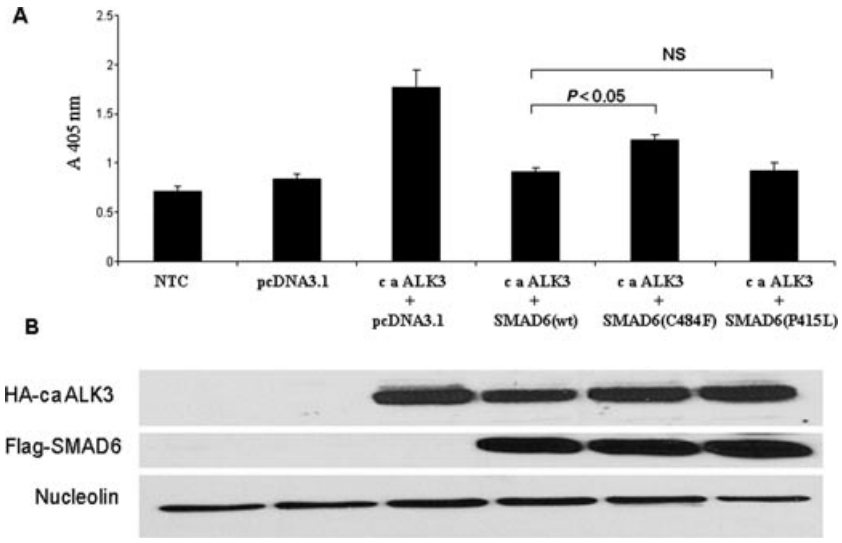

Figure 5. Inhibitory effects of SMAD6 variants on BMP signaling monitored via alkaline phosphatase (ALP) activity. **A**: ALP activity of constitutively active BMPR1A (caBMPR1A)-transfected C2C12 cells with and without SMAD6c variants was measured as absorbance at 405 nm per mg/ml protein. **B**: Immunoblots show levels of expression of caBMPR1A and SMAD6 protein; endogenous nucleolin was used as a loading control. SMAD6 (pC485F) showed significant loss of inhibitory activity, whereas there was no detectable effect of SMAD6 (pP415L).

variant allele was found to have a bicuspid aortic valve with mild aortic stenosis and aortic coarctation at the age of 30 years in the course of investigation for hypertension, and the coarctation was repaired. He subsequently developed significant aortic stenosis and underwent aortic valve replacement and rerepair of the aortic arch. At his second operation, it was noted that the transverse aortic arch, proximal to and distant from the previous conduit, was heavily calcified. It is possible that this is a consequence of the reduced efficiency of the p.C484F mutant SMAD6 in inhibiting osteogenic potential (Fig. 5). There was no evidence of inappropriate calcification in noncardiovascular tissues.

The other functionally significant SMAD6 variant we discovered (p.P415L) was present in a patient who presented with a heart murmur at 18 months and was found to have a bicuspid aortic valve with moderate aortic stenosis. There was no evidence of coarctation. Both patients carrying functionally significant *SMAD6* variants had bicuspid aortic valves, the commonest cardiovascular malformation, occurring in approximately 1% of the adult population. There is a phenotypic spectrum in this condition dependent on the degree of valvular malformation, ranging from severe aortic stenosis in the neonatal period to the usual presentation either as an asymptomatic murmur or established aortic stenosis in adult life. As this cohort of CVM cases was mainly recruited through a pediatric cardiology service, the numbers with bicuspid aortic valve were relatively small (24/436). It will be interesting to test whether *SMAD6* mutations are over-represented in a larger cohort of CVM patients with bicuspid aortic valves and other aortic malformations.

In the *Smad6* knockout mouse originally described by Galvin et al. (2000), multiple cardiovascular developmental abnormalities, including hyperplastic thickening of the cardiac valves and aortic ossification, are present. The association of *SMAD6* mutations with an aortic stenosis phenotype in the patients described in this study is entirely consistent with those observations and with a similar role for SMAD6 in human cardiovascular development. Our findings are also consistent with the expression of SMAD6 in the cardiac valves and outflow tract, which continues into adult life in mouse [Galvin et al., 2000], and with a recent clinical phenotypic study showing that reduced SMAD6 expression was associated with calcification of the aortic valve [Ankeny et al., 2011].

Exon-focused sequencing of two other genes (*BMPR2* and *BMPR1A*) in the BMP signaling pathway revealed no nonsynonymous variants. We therefore conclude that such variants are uncommon in these genes in CVM patients; however, sequencing of much larger number of cases would be required to exclude prevalences of 1–2%. On the basis of information from mouse models, there is a significant involvement of other BMP- and transforming growth factor β (TGFβ)-related genes in cardiac development that also warrants further investigation in congenital heart disease [Arthur and Bamforth, 2011; Wang et al., 2011]. Variants in some of these genes have already been shown to be associated with cardiovascular abnormalities in human studies, for example, *Nodal*, *GDF1*, *TGFβ3*, and *BMPR2* [Beffagna et al., 2005; Karkera et al., 2007; Mohapatra et al., 2009; Roberts et al., 2004; Roessler et al., 2009]. In one interesting case, a dominant-negative form of the BMP receptor ALK2 was found in a patient with endocardial cushion defects [Smith et al., 2009].

Although a few families have been described in which CVM segregates in a Mendelian fashion, for example, due to mutations in cardiac transcription factors [Garg et al., 2003; Schott et al., 1998], these families are exceptional and usually the inheritance pattern is less obvious; indeed, in the majority of cases, there is a single affected individual in a family. On analysis of pedigrees, however, the risk is clearly increased in the relatives of affected individuals, indicating a significant genetic contribution [Burn et al., 1998; Oyen et al., 2009]. These recurrence risks are compatible with the presence of multiple genetic risk variants of incomplete penetrance, likely interacting with environmental factors. We, and others, have already shown that incompletely penetrant alleles in key genes can predispose to CVM [Goldmuntz et al., 2001; Griffin et al., 2010; McElhinney et al., 2003; Sperling et al., 2005]. Future studies utilizing the rapidly increasing power of genome sequencing technologies to interrogate a much wider range of candidate genes, and eventually the whole exome, for rare variants that predispose to CVM will be of great interest. In the context of SMAD6, our results clearly demonstrate