VACTERL/caudal regression/Currarino syndrome-like malformations in mice with mutation in the proprotein convertase Pcsk5

Dorota Szumska,1,10 Guido Pieles,1,10 Rachid Essalmani,2 Michal Bilski,1 Daniel Mesnard,3 Kulvinder Kaur,4 Angela Franklyn,1 Kamel El Omari,4 Joanna Jefferis,1 Jamie Bentham,1 Jennifer M. Taylor,4 Jurgen E. Schneider,1 Sebastian J. Arnold,4 Paul Johnson,5 Zuzanna Tymowska-Lalanne,6 Dave Stammers,4 Kieran Clarke,7 Stefan Neubauer,1 Andrew Morris,4 Steve D. Brown,6 Charles Shaw-Smith,8 Armando Cama,9 Valeria Capra,9 Jiannis Ragoussis,4 Daniel Constam,3 Nabil G. Seidah,2 Annik Prat,2 and Shoumo Bhattacharya1,11

1Department of Cardiovascular Medicine and Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, United Kingdom; 2Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montreal, Montreal, Quebec H2W 1R7, Canada; 3Swiss Institute for Experimental Cancer Research-Ecole Polytechnique Federale de Lausanne (ISREC-EPFL), Swiss Federal Institute of Technology, CH-1066 Epalinges, Lausanne, Switzerland; 4Wellcome Trust Centre for Human Genetics, Oxford OX3 7BN, United Kingdom; 5Nuffield Department of Surgery, University of Oxford, Oxford OX3 9DU, United Kingdom; 6Medical Research Council (MRC) Mammalian Genetics Unit, Harwell OX11 0RD, United Kingdom; 7Department of Physiology, Anatomy, and Genetics, University of Oxford, Oxford OX1 3QX, United Kingdom; 8Department of Medical Genetics, Addenbrooke’s Hospital, Cambridge CB2 0XY, United Kingdom; 9Unità Operativa Neurochirurgia, Istituto G. Gaslini, 16148 Genova, Italy

We have identified an ethylnitrosourea (ENU)-induced recessive mouse mutation (Vcc) with a pleiotropic phenotype that includes cardiac, tracheoesophageal, anorectal, anteroposterior patterning defects, exomphalos, hindlimb hypoplasia, a presacral mass, renal and palatal agenesis, and pulmonary hypoplasia. It results from a C470R mutation in the proprotein convertase PCSK5 (PC5/6). Compound mutants (Pcsk5Vcc/null) completely recapitulate the Pcsk5Vcc/Vcc phenotype, as does an epiblast-specific conditional deletion of Pcsk5. The C470R mutation ablates a disulfide bond in the P domain, and blocks export from the endoplasmic reticulum and proprotein convertase activity. We show that GDF11 is cleaved and activated by PCSK5A, but not by PCSK5A-C470R, and that Gdf11-deficient embryos, in addition to having anteroposterior patterning defects and renal and palatal agenesis, also have a presacral mass, anorectal malformation, and exomphalos. Pcsk5 mutation results in abnormal expression of several paralogous Hox genes (Hoxa, Hoxc, and Hoxd), and of Mnx1 (Hlxb9). These include known Gdf11 targets, and are necessary for caudal embryo development. We identified nonsynonymous mutations in PCSK5 in patients with VACTERL (vertebral, anorectal, cardiac, tracheoesophageal, renal, limb malformation OMIM 192350) and caudal regression syndrome, the phenotypic features of which resemble the mouse mutation. We propose that Pcsk5, at least in part via GDF11, coordinately regulates caudal Hox paralogs, to control anteroposterior patterning, nephrogenesis, skeletal, and anorectal development.

[Keywords: Mouse; proprotein convertase; Gdf11; Hox; VACTERL]

Supplemental material is available at http://www.genesdev.org.

Received March 12, 2008; revised version accepted April 1, 2008.
mutagenesis, and make no a priori assumptions about the nature of the genes involved in development. The point mutagen ethynitrosourea [ENU] has been successfully used in screens for mouse developmental malformations [Herron et al. 2002; Yu et al. 2004; Bogani et al. 2005; Garcia-Garcia et al. 2005], and has certain advantages over genotype-driven knockout approaches. ENU results in loss-of-function, partial loss-of-function [i.e., hypomorphic] and gain-of-function alleles that are similar to the spectrum observed in the naturally occurring mutants causing human disease. These alleles can also allow in vivo investigation of the functional consequences of abnormal protein structure [for review, see Papathanasiou and Goodnow 2005].

Given an appropriate screening method, a phenotype-driven screen could be used to identify the genetic mechanisms underlying any developmental malformation. We established a recessive three-generation ENU mutagenesis screen that uses high-throughput magnetic resonance imaging [MRI] of mouse embryos [Schneider et al. 2004] at 15.5 d post-coitum [dpc] as the primary screening technique to identify cardiac developmental malformations. In this screen we identified a recessive mutation [Vcc] with cardiac, tracheoesophageal, anal-rectal, axial skeletal anteroposterior patterning defects, limb malformations, presacral mass, renal and palatal agenesis, and pulmonary hypoplasia. This pleiotropic phenotype resembles those observed in certain human syndromes: VACTERL (vertebral, anal-rectal, cardiac, tracheoesophageal, renal, limb malformation OMIM 192350), caudal regression syndrome (CRS, OMIM 600,145), and Currarino Syndrome (CS, OMIM 176450).

Results

Vcc: a novel recessive ENU mouse mutant with features of VACTERL/CRS/CS

In 11 of 57 embryos that were progeny of a G1 founder male [RECC/19], and in six of 19 embryos from tested G2 males we identified consistent externally visible features including short hindlimbs, absent tail, and exomphalos [Fig. 1]. In 15 embryos analyzed by MRI we observed palatal agenesis, abnormal tracheoesophageal septation, cardiac malformation, and small lungs [Figs. 1, 2]. Cardiac malformations included dextroposition [eight of 15], atrial septal defects [six of 15], ventricular septal defects [15 of 15], double-outlet right ventricle [10 of 15], common arterial trunk [five of 15], hypoplastic arterial duct [two of 15], aortic vascular ring [three of 15], and right-sided aortic arch [two of 15]. Measurement of lung volumes showed that the left lung was significantly hypoplastic [Fig. 2]. All embryos had a complete absence of kidneys [although adrenal glands were present], and had a mass protruding ventrally from the caudal end of the spinal cord and vertebral column [Fig. 3]. This mass distorted normal anal-rectal and bladder anatomy, and three-dimensional [3D] reconstruction showed that the anus and rectum could not be identified [Fig. 3]. Examination of skeletal morphology [two mutant embryos studied]
showed absence of caudal and sacral vertebrae, normal lumbar and cervical vertebral numbers, and 18 instead of 13 thoracic vertebrae (Fig. 4), giving a total of 31 instead of the normal 26 presacral vertebrae. Mutant embryos also had increased numbers of true ribs, and lacked a xiphisternum. The pelvic bones, femur, and tibia were severely hypoplastic. The patella, fibula, and bones of the foot were not visible. The forelimbs were mildly hypoplastic.

**Vcc results from a mutation in Pcsk5 with potential structural consequences**

We mapped a minimal homozygous segregating interval to between D19MIT128 and rs13483551 (Chr 19: 17317664–18953883). Two genes in this interval (Gcnt1 and Trpm6) could be excluded as null mutations are viable (Ellies et al. 1998; Walder et al. 2002). We sequenced the exons of all other genes in this interval, and identified a homozygous T/C transition in Pcsk5 exon 11 (Supplemental Fig. 1). The mutation predicted a C470R amino acid change. PCSK5 is a proprotein convertase, a class of enzymes that cleave proproteins and prohormones at the consensus sequence [K/R]-[Xn]-[K/R]↓ (for review, see Seidah and Chretien 1999). PCSK5 has two splice isoforms, A and B (Supplemental Fig. 1). PCSK5A (exons 1–21a) is soluble and is sorted to regulated secretory granules, whereas PCSK5B (exons 1–20, 21b, and 22–38) is membrane-bound, and located in the Golgi apparatus (De Bie et al. 1996; Xiang et al. 2000). The C470R mutation would be predicted to affect both splice isoforms. By analogy with FURIN, PCSK5-C470 is in the P domain, and is predicted to form a disulfide bond with C496 [Henrich et al. 2005]. Molecular modeling of FURIN reveals that an analogous mutation (C450R) would result in displacement of the loop (471–482) away from P/H92521a (Supplemental Fig. 1).

**Vcc does not complement a Pcsk5 deletion allele**

To determine if the Vcc mutation complements the loss of Pcsk5, we crossed Vcc females to males bearing a targeted deletion of Pcsk5 exon 1 (Pcsk5Δ1) (Essalmani et al. 2008) and examined the phenotype of double and single heterozygotes by magnetic resonance imaging. We found that double heterozygotes [Pcsk5Vcc/Δ1] completely recapitulated the Vcc/Vcc phenotype in all embryos studied (eight of eight) (Supplemental Fig. 2). All double heterozygote embryos had hypoplastic hindlimbs, absent tail, exomphalos, presacral mass, palatal agenesis, cardiac malformations, and an abnormal VACTERL/CRS/CS-like phenotype with Pcsk5 mutation.

**Figure 2.** Cardiac and pulmonary malformations at 15.5 dpc. (a) MRI transverse section through a control embryo (at 15.5 dpc) showing a normal heart. The right and left atria and ventricles (RA, LA, RV, LV), mitral valve [MV], primary atrial septum [PAS], and interventricular septum [IVS] are indicated. [a′] Corresponding section of a mutant littermate Vcc/Vcc embryo showing an ostium primum atrial septal defect (ASD) and common atrioventricular valve [CAVV]. (b) Three-dimensional reconstruction of a control heart, left anterior oblique view. The aorta (Ao) arises from the left ventricle (LV), and the main pulmonary artery (PA) rises from the right ventricle (RV). The aortic arch passes to the left of the trachea, over the left main bronchus. (b′) Corresponding reconstruction of a mutant littermate Vcc/Vcc heart showing a ventricular septal defect (VSD), a common arterial trunk (CAT) arising from the right ventricle (RV), giving rise to both cranial and pulmonary arteries, and an aortic vascular ring (AoVR). The trachea is not separated from the esophagus (Tr-O). (c,c′) Three-dimensional reconstruction of lungs from wild-type (wt) and littermate mutant [Vcc/Vcc] embryos. The cranial (Cr), middle (mi), caudal (Ca), and accessory (Ac) lobes of the right lung and the left lung (LL) are indicated. (d) Lung volumes (mean ± SEM, corrected for embryo volume) are shown for control left and right lungs (Con-LL, Con-RL), and littermate mutant Vcc/Vcc left and right lungs (Mut-LL, Mut-RL). The mutant left lung is significantly smaller than the control (t-test, P = 0.003). Axes are anterior (A), posterior (P), right (R), left (L), dorsal (D), and ventral (V). Bars, 0.5 mm.
and tracheoesophageal malformations, pulmonary hypoplasia, and renal agenesis. The cardiac malformations included dextroposition (two of eight), ventricular septal defect (eight of eight), atrial septal defect (three of eight), double-outlet right ventricle (six of eight), common arterial trunk (two of eight), and aortic vascular ring (one of eight). No abnormalities were observed in single heterozygous control embryos. These results show that Vcc does not complement Pcsk5Δ1.

Epiblast-specific deletion of Pcsk5 recapitulates the Vcc phenotype

Pcsk5 is expressed in maternal-derived, hypoblast-derived, and epiblast-derived tissues (Constam et al. 1996; Rancourt and Rancourt 1997). To determine the role of epiblastic Pcsk5 in organogenesis and patterning we made use of a Pcsk5flox allele that deletes exon 1 by Cre-mediated recombination (Essalmani et al. 2008). We used this allele to delete Pcsk5 specifically in the epiblast using Meox2Cre and studied the phenotype of embryos (Meox2Cre; Pcsk5Δ1/flx) at 15.5 dpc using MRI (Supplemental Fig. 3). We found that this recapitulated the Vcc/Vcc phenotype, but with reduced penetrance, likely reflecting incomplete deletion of Pcsk5. Meox2Cre; Pcsk5Δ1/flx embryos had hypoplastic hindlimbs (four of four), absent or hypoplastic tail (four of four), cardiac malformations (four of four), palatal agenesis (three of four), tracheoesophageal malformation (two of four), pulmonary hypoplasia affecting left lung (two of four), exomphalos (three of four), and renal agenesis (three of four). The cardiac malformations included dextroposition (one of four), transposition of great arteries (one of four), double-outlet right ventricle (three of four), ventricular septal defect (four of four), atrial septal defect (two of four), right-sided aortic arches (two of four), and aortic vascular ring (one of four). A presacral mass distorting anorectal and bladder anatomy was observed in the two embryos that lacked a visible external tail. Meox2Cre; Pcsk5Δ1/flx embryos also had axial and appendicular skeletal defects similar to Vcc/Vcc (Essalmani et al. 2008).

PCSK5A C470R mutation affects its secretion and localization

To investigate the functional consequences of the C470R mutation we transfected PCSK5A-Flag and PCSK5A-C470R-Flag-expressing plasmids into COS-1

Figure 3. Visceral malformations. [a] MRI transverse sections through the abdomen of a wild-type embryo (wt) at 15.5 dpc, showing the normal kidneys (LK, left kidney), ureter (LU, left ureter) and adrenals (LAd, left adrenal). [a’] Corresponding sections through a mutant littermate Vcc/Vcc embryo. The kidneys and ureter could not be identified. The pancreas (P) is indicated. [b] MRI sagittal sections through a 15.5-dpc control embryo showing the normal urinary bladder (UB), urethra (U), liver (Li), and vertebral column (VC). The tail (T) is indicated. [b’] Corresponding section through a mutant littermate Vcc/Vcc embryo. A presacral mass (PM) is seen between the vertebral column and the bladder. The liver herniates through the umbilicus forming an exomphalos (Ex). [c] Three-dimensional reconstruction of a control embryo showing the spinal cord (SC), gut (G), urinary bladder (UB), urethra (U), and rectum (R). [c’] Corresponding reconstruction of a mutant Vcc/Vcc embryo showing that the presacral mass (PM) is connected to the spinal cord, and its relation to the bladder and gut. The rectum could not be defined. [d] Sagittal histological section of a 13.5-dpc wild-type embryo showing the vertebral column (VC) and the spinal cord (SC) entering the tail (T). [d’] Corresponding section of a littermate mutant Vcc/Vcc embryo showing the vertebral column and spinal cord entering the presacral mass (PM). Axes are anterior (A), posterior (P), right (R), left (L), dorsal (D), and ventral (V). Bars, 0.5 mm.
cells, and examined the secretion and immunolocalization of the Flag-tagged peptides (Fig. 5). Immunoblotting and pulse-label radioimmunoprecipitation experiments indicated that both Flag-tagged and untagged versions of PCSK5A (wild type) are efficiently synthesized and secreted into the medium. Although tagged and untagged versions PCSK5A-C470R were also efficiently synthesized, they could not be detected in the conditioned medium. Immunofluorescence experiments showed that whereas PCSK5A-Flag is localized to the trans-Golgi network, PCSK5A-C470R-Flag localizes instead to the endoplasmic reticulum (Fig. 5).

**PCSK5A C470R mutation affects its enzymatic activity**

We next used cotransfection assays to examine the effect of the mutation on the cleavage of two PCSK5 substrates, BMP4 (Cui et al. 1998), and LEFTY1 (Ulloa et al. 2001). These experiments showed that whereas PCSK5A efficiently cleaves BMP4 and LEFTY1 propeptides to their mature forms, the mutant PCSK5A-C470R does not (Fig. 5). We also monitored the enzymatic activity of the conditioned medium from transfected cells using a fluorogenic peptide assay. This showed that while conditioned medium from cells transfected with wild-type PCSK5A plasmid had in vitro enzymatic activity, the activity of conditioned medium from cells transfected with PCSK5A-C470R plasmid was identical to that of vector transfected cells [Supplemental Fig. 4].

**Gdf11 deletion partially phenocopies Vcc**

Certain features of the Vcc mutant [palatal agenesis, renal agenesis, increased numbers of thoracic vertebrae to 18, increased numbers of true ribs, absent tail] phenocopy the loss of Gdf11 (McPherron et al. 1999; Esqueila and Lee 2003). We examined Gdf11−/− embryos at 15.5 dpc using MRI, and confirmed that they had absent tail [five of five], palatal agenesis [five of five], and renal agen-
esis [five of five]. In addition, we found that these embryos had either an intra-abdominal [three of five] or an extra-abdominal [two of five] mass arising from the spinal cord, exomphalos [two of five], and abnormal anorectal anatomy [five of five] [Fig. 6]. We found no evidence of cardiac or tracheoesophageal malformation or lung or hindlimb hypoplasia in these embryos.

GDF11 is activated and cleaved by PCSK5A

GDF11 is a TGFβ superfamily molecule [McPherron et al. 1999], and sequence analysis reveals that it contains a consensus proprotein convertase cleavage site [Duckert et al. 2004], suggesting that PCSK5 may cleave the GDF11 propeptide to its mature form. We used a cotransfection luciferase assay that detects activated GDF11 to determine if it can be activated by cotransfected PCSK5A [Yan et al. 2002; Andersson et al. 2006]. In this assay we found that transfection of GDF11 alone into 293T cells activated the reporter, likely reflecting the presence of an endogenous proprotein convertase [Fig. 6]. This activity was increased threefold by cotransfecting in PCSK5A-Flag. In contrast, cotransfection of PCSK5A-C470R-Flag did not affect reporter activity. We also assayed the ability of conditioned medium from cells transfected with PCSK5A-Flag and PCSK5A-C470R-Flag to cleave a GDF11-derived peptide containing the putative cleavage site. We found that conditioned medium from PCSK5A-Flag transfected cells achieved a percent cleavage of 58.4% [mean of two independent experiments], whereas no significant cleavage was observed with conditioned medium from PCSK5A-C470R-Flag transfected cells [Fig. 6]. Taken together, these results indicate that PCSK5A can cleave and activate GDF11, and that the C470R mutation ablates this function.

The Vce mutation does not affect embryonic Pcsk5 or Gdf11 expression

Endogenous Pcsk5 transcript could be detected in wild-type embryos, most strongly in the caudal-most somites, to a reduced extent in more anterior somites, and also strongly in the apical ectodermal ridges of the forelimb, and hindlimb buds [three of three examined] [Fig. 7], consistent with previous reports [Constam et al. 1996; Es-salmani et al. 2006]. This distribution was unchanged in
littermate mutant Vcc/Vcc embryos (three of three examined) [Fig. 7]. We were unable to detect endogenous PCSK5 protein from mouse embryos using five commercial antibodies by Western blotting (Supplementary Fig. 7). Endogenous Gdf11 transcript could be detected in wild-type embryos in the tailbud, and in the forelimb bud and hindlimb bud, consistent with previous observations [McPherron et al. 1999; Nakashima et al. 1999]. This distribution was unchanged in littermate mutant Vcc/Vcc embryos (three of three examined) [Fig. 7].

The Vcc mutation results in abnormal Hox and Mnx1 gene expression

Gdf11 deletion is associated with abnormal Hoxc10 and Hoxc11 expression [McPherron et al. 1999]. We therefore explored the idea that Pcsk5 controls Hox expression using in situ hybridization [Fig. 7]. We found that while Hoxc10 was expressed in the caudal region of wild-type embryos, it was almost absent in littermate Vcc/Vcc mutants [three of three mutants studied] [Fig. 7]. Hoxa11 was expressed in the forelimb and hindlimb buds in wild-type controls, but was markedly reduced in the littermate mutant embryos [three of three mutants studied]. Hoxa10 and Hoxd11 were expressed in the tailbud region, forelimb bud, and hindlimb bud in wild-type controls but markedly reduced in the littermate mutant embryos [three of three mutants studied] [Fig. 7].

Using an unbiased microarray approach (Supplemental Fig. 5) we also found that the expression of several Hox genes was significantly reduced in Vcc/Vcc mutants. These included Hoxc9, Hoxc10, Hoxc9, Hoxd10, and Hoxd12. In addition, the expression of Hoxa11os [a non-coding transcript from the opposite strand of the Hoxa locus] [Hsieh-Li et al. 1995] was also significantly reduced. Using in situ hybridization we found that the Hoxa11os was expressed in tailbud and forelimb bud in wild-type, but was markedly reduced in the littermate mutant embryos. Hoxd10 was expressed in tailbud, forelimb bud, and hindlimb bud in the wild-type, but markedly reduced in tailbud and hindlimb bud in the littermate mutant embryos. Hoxd12 was expressed in tailbud, forelimb bud, and hindlimb bud in the wild-type, but undetectable in littermate mutant embryos (three of three mutants studied) [Fig. 7].

In addition to Hox genes, we found that the homeobox-like gene Mnx1 [Hlxb9; Hb9], which is expressed in the motorneurons and specifies their identity; and the human ortholog of which is mutated in CS [Ross et al. 1998; Arber et al. 1999; Harrison et al. 1999; Li et al. 1999; Lynch et al. 2000], was ectopically expressed in the ventral aspect of the tail bud (three of three mutants studied) [Fig. 7]. In contrast, no differences were observed...
in expression of other caudal markers such as T, Tbx6 (three of three mutants studied) [Fig. 7].

**Nonsynonymous mutations in VACTERL/CRS patients**

The phenotypic features of this mouse mutant resemble an overlap between VATER/VACTERL association, CRS, and CS. We studied European (Italian and British) patients with features of VACTERL/CRS/CS for mutations in the open reading frame of PCSK5 using amplicon DNA melting analysis (Herrmann et al. 2006) followed by sequencing. VACTERL is diagnosed when three or more features comprising the association are present in a patient [Khoury et al. 1983]. In 36 patients with a VACTERL score ≥3 [assigning a score of 1 for each VACTERL component] we identified four patients with nonsynonymous variants in PCSK5B (two with E1575K,
that an epiblast-specific conditional deletion of Pcsk5 exon1 (Meox2Cre; Pcsk5\(^{VA/VA}\)\(^{VA}\)) recapitulates the cardiac, tracheoesophageal, and anorectal malformations, hind-limb hypoplasia, presacral mass, exomphalos, renal and palatal agenesis, and pulmonary hypoplasia phenotype. Axial skeletal malformations and renal agenesis in these conditionally deleted embryos have also been reported independently [Essalmani et al. 2008]. These results indicate that Pcsk5 functions within the epiblast are necessary for anteroposterior patterning and organogenesis. Notably, embryos with Pcsk5\(^{Vcc/Vcc}\), Pcsk5\(^{VA/VA}\)\(^{VA}\), Pcsk5\(^{Vcc/\Delta1}\), and Pcsk5\(^{VA/\Delta1}\) genotypes all survive until 15.5 dpc and beyond [Essalmani et al. 2008; this study]. However the Pcsk5 exon 4 deletion (Pcsk5\(^{VA/\Delta4}\)) results in embryonic lethality by 4–7 dpc [Essalmani et al. 2006]. The mechanism for this earlier embryonic lethality in Pcsk5\(^{VA/\Delta4}\) is unclear at present, but may reflect the differing genetic backgrounds of the mice. Alternatively, it is possible that secreted PCSK5A4 peptide titrates a PCSK5-interacting protein [Essalmani et al. 2006].

The predicted structure of the protein resulting from the mutated gene indicates that the C470R mutation will likely disrupt a predicted disulfide bond in the P domain. The P domain in the related molecule FURIN is an eight-stranded β-sandwich that has interdomain contacts with the catalytic domain, and is conserved in PCSK1–7. The predicted effect of the analogous mutation in the FURIN structure is to displace the loop (471–482) away from P\(^{I9}\). The P domain is thought to stabilize the catalytic domain, and the propeptide may bind to a crevice formed by the P domain and the catalytic site [Henrich et al. 2003]. Thus, the loss of catalytic activity observed in our in vitro experiments (on BMP4, LEFTY1, and GDF11) is likely a consequence of abnormal P domain folding. As the P domain is necessary for the intramolecular cleavage and normal trafficking of proprotein convertases through the secretory pathway [Zhou et al. 1998; Ueda et al. 2003], it is likely that the abnormal trafficking of the PCSK5A-C470R mutant observed in our studies [reduced secretion, and ER retention] is a consequence of the perturbed P domain structure and reduced catalytic activity. Although these results suggest that loss of PCSK5A catalytic activity is the mechanism for the Vcc phenotype, an alternative explanation is that the C470R mutation in vivo may simply result in protein degradation as a consequence of misfolding. A limitation of our studies is that available antibodies do not allow us to detect endogenous PCSK5 protein from mouse embryos, and we are unable to present at present to distinguish between these two alternative explanations.

Genetic evidence indicates that certain PCSK5 targets such as BMP4 [Cui et al. 1998] and LEFTY1 [Ulloa et al. 2001] are necessary for normal cardiac development [Meno et al. 1998; Jiao et al. 2003; Liu et al. 2004; Goldman et al. 2006]. Our studies showed that PCSK5A-C470R does not cleave either BMP4 or LEFTY1 in co-transfection assays. BMP4 deficiency is associated with cardiac abnormalities that include common arterial trunk, and atriocentral septation defects [Jiao et al.
Szumska et al.

2003; Liu et al. 2004), both abnormalities being observed in Pcsk5 mutant embryos. Importantly, BMP4 cleavage is essential for ventral body wall closure (mutation resulting in omphalocoelexomphalos), and cardiac development (Goldman et al. 2006). These features [exomphalos and abnormal cardiac development] were observed in the Pcsk5 mutants. Cardiac malformations in LEFTY1 deficiency include double-outlet right ventricle, transposition of great arteries, atrioventricular septal defect, and right aortic arch (Meno et al. 1998). These malformations were also observed in Pcsk5 mutants. Thus it is possible that cardiac malformations in the Vcc mutant arise, at least in part, as a result of abnormal BMP4 or LEFTY1 cleavage.

Certain features of the Pcsk5Vcc/Vcc mutant—palatal agenesis, renal agenesis, increased numbers of thoracic vertebrae to 18, increased numbers of true ribs—pheno-
copy the loss of Gdf11 (McPherron et al. 1999; Esquela and Lee 2003). Our results presented here extend the known phenotypic features of Gdf11 deficiency, and show that there are further points of similarity with Pcsk5 deficiency, i.e., Gdf11 deficiency also results in a presacral mass arising from the spinal cord, exomphalos, and anorectal malformation. We showed that GDF11 peptide can be cleaved by PCSK5A, that this activates GDF11-mediated signaling, and that Gdf11 expression is not deficient in the Pcsk5Vcc/Vcc mutant. Moreover, Gdf11 and Pcsk5 are expressed in close physical proximity in the developing embryo. Gdf11 is expressed in the tailbud and neural tube, whereas Pcsk5 is expressed in the adjacent caudal somites (Constam et al. 1996; McPherron et al. 1999; Nakashima et al. 1999; Essalmani et al. 2006; this study). Similarly in limb buds, while Gdf11 is expressed in the mesenchyme, Pcsk5 mRNA localizes to the overlying apical ectodermal ridge. This situation is reminiscent of the activation of the related NODAL precursor in the epiblast by the proprotein convertases FURIN and PACE4, which are provided by cells in the adjacent extraembryonic ectoderm and visceral endoderm (Beck et al. 2002; Mesnard et al. 2006).

GDF11 is a TGFβ superfamily molecule (McPherron et al. 1999), and analysis of both human and mouse GDF11 peptides indicates that they have a completely conserved consensus proprotein convertase cleavage site (after residue 298 in the human peptide, i.e., NTKRSSRR4NL) (Duckert et al. 2004). This supports the idea that both mouse and human GDF11 are PCSK5A targets. Independent experiments have shown that selectivity of PCSK5A for GDF11 is mediated by the Asn residue immediately following the cleavage site, and that other proprotein convertases [FURIN, PCSK7 [PC7] or PCSK6 [PACE4]] will not cleave GDF11 [Essalmani et al. 2008]. Taken together, these results indicate that Pcsk5 functions at least in part by activating GDF11 signaling in the developing embryo. Consistent with this, mutation in Gdf11, in the type II receptor Acvr2b, and the type I receptor Alk5, are associated with a caudal phenotype involving increased numbers of thoracic vertebrae, and renal agenesis, and are associated with abnormal expression of Hoxc6, Hoxc8, Hoxc10, and Hoxc11 (Oh and Li 1997; McPherron et al. 1999; Oh et al. 2002; Esquela and Lee 2003; Andersson et al. 2006).

The regulation of Hox expression is complex, involving GDF11 and BMPs [McPherron et al. 1999; Li and Cao 2006], TGFβ receptors [Oh and Li 1997; Oh et al. 2002; Andersson et al. 2006], BMP receptors [Ovchinnikov et al. 2006], retinoic acid receptors [Daftary and Taylor 2006], chromatin modifying Polycomb/Trithorax genes [Renge and Paro 2007], and noncoding transcripts that arise at the Hox locus [Rinn et al. 2007; Sessa et al. 2007]. Genetic evidence shows that Hox10acd is necessary for the suppression of thoracic ribs on lumbar and sacral vertebrae, and formation of the hindlimb stylopod (femur, patella) [Wellik and Capecchi 2003]. Hox11acd is necessary for the formation of the sacrum and the hindlimb zeugopod (tibia, fibula) [Wellik and Capecchi 2003], and the metanephric kidney [Wellik et al. 2002]. Hoxd12 is necessary for normal anorectal development (Kondo et al. 1996). Our experiments establish a new mechanism for the coordinated regulation of paralogous caudal Hox gene expression. We observed reduction of Hox10acd expression in Pcsk5 mutant embryos. This could explain the increased numbers of thoracic vertebrae, hypoplastic femur, and absent patella. The reduction of Hox11ad in Pcsk5 mutant embryos [most marked in the caudal region and the hindlimb bud], could explain the absence of sacrum formation, abnormal tibia and fibula patterning, and renal agenesis. The reduction of Hoxd12 would explain the anorectal malformations. The relatively normal expression of Tbx6 and T, genes also important in renal, vertebral, and caudal patterning [Showell et al. 2004], suggest that these are unlikely to contribute to the Pcsk5 mutant phenotype. In addition, our results show that Pcsk5 also regulates expression of a noncoding transcript Hoxa11os [Hsieh-Li et al. 1995]. While the function of this transcript is not known, other noncoding Hox locus transcripts have been shown to regulate Hox expression [Rinn et al. 2007].

Our results also show that Mnx1 (Hlxb9, Hb9), the mouse homolog of HLXB9, is misexpressed in the tail bud region of Pcsk5 mutant embryos. Mnx1 specifies motor neurons and the dorsal pancreas [Arber et al. 1999; Harrison et al. 1999; Li et al. 1999], and ectopic expression triggers motorneuron differentiation [Arber et al. 1999]. The ectopic Mnx1 expression observed could explain the presacral neural-derived mass observed in RECC19 embryos. A characteristic feature of CS is this presacral mass [Carrubba et al. 1981; Lynch et al. 2000], and it is notable that HLXB9 mutations are associated with CS/Autosomal-dominant sacral agenesis [Ross et al. 1998; Lynch et al. 2000], suggesting a link.

The features observed in the mouse embryos recapitulate many aspects of the VACTERL association, and also of CRS and CS, syndromes that have significant overlap with VACTERL. Specifically, the mouse mutant has vertebral, anorectal, cardiac, tracheoesophageal, renal, and limb malformations observed in VACTERL association. The mouse mutant also recapitulates anomalies associated with CRS—i.e., sacral agenesis, spinal cord anomalies, and pulmonary hypoplasia. In addition, it recapitu-
lates the presacral mass, a feature specific to CS. These shared features suggested that Pcsk5 may play a role in these human syndromes. Our preliminary investigations in this regard support this idea as they identify certain heterozygous variants in evolutionarily conserved residues of PCSK5. These variants are present in patients with VACTERL/CRS phenotypes in humans but are absent in ethnically matched controls indicating that they are unlikely to be common polymorphisms. A confounding factor is that the mutations are inherited from apparently normal parents. This raises the possibility that the phenotype results from additional environmental modifiers such as maternal diabetes (Koussoff 1999, Aberg et al. 2001, Loffredo et al. 2001), and genetic modifiers; e.g., within the PCSK5 genetic network. The functional significance of these mutations is unclear at present, and further work is necessary to determine this.

Taken together, these studies extend our understanding of the role of proprotein convertases in the patterning of the mammalian embryo. Other members of the Pcsk family that have been shown to control embryonic patterning are Pcsk6 (SPC4/PACE4), which controls left-right patterning and anterior central nervous system development (Constam and Robertson 2000a), and Furin (Pcsk3/PACE/SPC1), which controls cardiac tube fusion and looping, embryo turning, ventral body wall closure, and yolk sac vasculature development (Roebrock et al. 1998; Constam and Robertson 2000b). In contrast, Pcsk5 has unique roles in controlling anteroposterior axial patterning, coordinated regulation of caudal Hox expression, neural and gut development, and nephrogenesis. Our results support the idea that activation of GDF11 by PCSK5 is likely necessary for normal anteroposterior patterning, nephrogenesis, and limb and anorectal development. They also suggest that activation of BMP4 and LEFTY1 by PCSK5 may play a role in normal cardiac development. The identification of nonsynonymous mutations in PCSK5B in patients with VACTERL and CRS suggests that the PCSK5 genetic network may play a role in the pathogenesis of these syndromes. In summary, our data establishes novel and pleiotropic functions for Pcsk5 in mammalian development and the coordinated expression of caudal Hox genes. We propose that Pcsk5, at least in part via GDF11, coordinate the expression of caudal Hox paralogs, to control anteroposterior patterning, nephrogenesis, and limb and anorectal development.

Materials and methods

Mice and embryos

Standard methods were used to create ENU mutant mice and genetically map the Vcc mutation (see the Supplemental Material). MRI and skeletal preparations were performed as described [Kaufman 1994, Schneider et al. 2004]. The generation of Pcsk5<sup>gef/c</sup> and Pcsk5<sup>−/−</sup> alleles is described elsewhere [Essalmani et al. 2008]. Gdf11 knockout mice were a kind gift from Se-Jin Lee (Johns Hopkins University, Baltimore, MD).

Molecular biology

Standard molecular cloning techniques were used [Ausubel et al. 1995].

Plasmids

We obtained mouse IMAGE clone 4036159 [CMV-PCSK5A] from Geneservice Ltd., and introduced a C-terminal Flag tag and the C470R mutation using PCR. PMT23-BMP2-Myc-tagged BMP4 was a kind gift from Tom Jessel (Columbia University). CMV-SPORT6-human-GDF11 plasmid was subcloned from a GDF11 plasmid (kind gift from Se-Jin Lee). AR3-lux reporter plasmid has been described previously [Yan et al. 2002]. LEFTY1 plasmid was a gift from H. Hamada (Osaka University). We obtained Hoxc10, Hoxa11, Max1, and Tbx6 mouse plasmids from Geneservice Ltd. [IMAGE: 30039851, 1095072, 40130560, 636895]. Hoxa10 is a gift from P. Gruss and A. Mansouri, Hoxd11 is a gift from D. Duboule, and Hoxd10 is a gift from D. Bogani. Hoxa11os plasmid was created by PCR and is common for opposite strand transcripts 3C, 23A, 59, and 40 [Hsieh-Li et al. 1995]. T plasmid was a gift from B. Hermann.

Cell-based assays

COS-1 and 293T cells were transfected with Fugene [Roche], and HEK293 cells were transfected with Effectene [Qiagen]. Monoclonal M2 anti-Flag antibody was obtained from Sigma, anti-myc mouse monoclonal 9E10 was obtained from Roche, goat anti-mouse HR conjugate was obtained from DAKO, LEFTY1 polyclonal rabbit antibody was obtained from Santa Cruz Biotechnologies [sc-7408], sheep anti-human TGN46 polyclonal antibody was obtained from Serotec, rabbit anti-calnexin antibody was obtained from Abcam [ab13504-100], and secondary Alexa Fluor488 goat anti-mouse-conjugated [green] and Alexa Fluor594 donkey anti-sheep-conjugated [red] antibodies were obtained from Invitrogen. Western blot analysis of whole-cell lysates and serum-free conditioned medium were performed as described [Beck et al. 2002]. Radioimmunoprecipitation assays were performed as described [Essalmani et al. 2006]. The luciferase reporter assay was performed as described [Yan et al. 2002, Andersson et al. 2006]. We performed immunofluorescence using standard techniques [Ausubel et al. 1995], and data were collected sequentially using a Zeiss confocal microscope. Peptide cleavage studies were performed as described [Essalmani et al. 2006, 2008].

In situ hybridization

In situ hybridization was performed as described previously [Bamforth et al. 2004].

Human mutations

Details are provided in the legend for Supplemental Figure 6.

Acknowledgments

These studies were funded by BHF grant PG/04/023/16761, the MRC, CIHR grant MOP 44363, Canada chair 201652, and Wellcome Trust Senior Research Fellowship 054528. We thank Se-Jin Lee for the gift of Gdf11 knockout mice, S. Wells, M. Cormack, and S. Polley for help with dissections and mouse colony maintenance; H. Barnes for help with MRI; L. Winchester for microarray analysis; D. Brooker for pyrosequencing; L. Buckingham and H. Butler for iPLEX assays; J. Majewska for help with 3D reconstructions; Z. Holloway for advice on antibodies and immunofluorescence; A. Pasquato for in vitro PCSK5 activity tests; D. Donnai, H. Murphy, M. Parker, E. Roper, E. Blair, H. Stewart, and M. Gatzoulis for providing patient samples; and all colleagues who generously sent us plas-
Szumska et al.

mids. S.B. is a Wellcome Trust Senior Research Fellow in Clinical Science.

References

Aber, A., Westbom, L., and Kallen, B. 2001. Congenital malformations among infants whose mothers had gestational diabetes or preexisting diabetes. Early Hum. Dev. 61: 85–95.

Andersson, O., Reissmann, E., and Ihanez, C.F. 2006. Growth differentiation factor 11 signals through the transforming growth factor-β receptor ALKS to regionalize the anterior–posterior axis. EMBO Rep. 7: 831–837.

Arber, S., Han, B., Mendelsohn, M., Smith, M.,Jessell, T.M., and Sockanathan, S. 1999. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron 23: 659–674.

Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D.,Seidman, J.G., Smith, J.A., and Struhl, K. 1995. Short protocols in molecular biology. John Wiley & Sons, Inc., New York.

Bamforth, S.D., Braagana, J., Farthing, C.R., Schneider, J.E., Broadbent, C., Michell, A.C., Clarke, K., Neubauer, S., Norris, D., Brown, N.A., et al. 2004. Cited2 controls left–right patterning and heart development through a Nodal–Pitx2c pathway. Nat. Genet. 36: 1189–1196.

Beck, S., Le Good, J.A., Guzman, M., Ben Haim, N., Roy, K., Beermann, F., and Constam, D.B. 2002. Extraembryonic proteases regulate Nodal signalling during gastrulation. Nat. Cell Biol. 4: 981–985.

Bogani, D., Willoughby, C., Davies, J., Kaur, K., Mirza, G., Paudyal, A., Haines, H., McKeone, R., Cadman, M., Pieles, G., et al. 2005. Dissecting the genetic complexity of human 6p deletion syndromes by using a region-specific, phenotype-driven mouse screen. Proc. Natl. Acad. Sci. 102: 12477–12482.

Constam, D.B. and Robertson, E.J. 2000a. SPC4/PACE4 regulates a TGFβ signaling network during axis formation. Genes & Dev. 14: 1146–1155.

Constam, D.B. and Robertson, E.J. 2000b. Tissue-specific requirements for the proprotein convertase furin/SPC1 during embryonic turning and heart looping. Development 127: 245–254.

Constam, D.B., Calfon, M., and Robertson, E.J. 1996. SPC4, SPC6, and the novel pro tease SPC7 are coexpressed with bone morphogenetic proteins at distinct sites during embryogenesis. J. Cell Biol. 134: 181–191.

Cui, Y., Jean, F., Thomas, G., and Christian, J.L. 1998. BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development. EMBO J. 17: 4735–4743.

Currarino, G., Cohn, D., and Votteler, T. 1981. Triad of anorectal, saleral, and presacral anomalies. AJR Am. J. Roentgenol. 137: 395–398.

Daftary, G.S. and Taylor, H.S. 2006. Endocrine regulation of HOX genes. Endocr. Rev. 27: 331–355.

De Bie, I., Marcinkiewicz, M., Malide, D., Lazure, C., Nakayama, K., Bendayan, M., and Seidah, N.G. 1996. The isoforms of proprotein convertase PCS are sorted to different subcellular compartments. J. Cell Biol. 135: 1261–1275.

Duckert, P., Brunak, S., and Blom, N. 2004. Prediction of proprotein convertase models based on the crystal structures of furin and/or PC6 during vertebrate embryonic development. EMBO J. 17: 4735–4743.

Ellies, L.G., Tsuboi, S., Petryniak, B., Lowe, J.B., Fukuda, M., and Marth, J.D. 1998. Core 2 oligosaccharide biosynthesis distinguishes between selectin ligands essential for leukocyte homing and inflammation. Immunity 9: 881–890.

Esquela, A.F. and Lee, S.J. 2003. Regulation of metastatic kid-
gene Hlxb9. Nat. Genet. 23: 67–70.
Liu, W., Selever, J., Wang, D., Lu, M.F., Moses, K.A., Schwartz, R.J., and Martin, J.F. 2004. Bmp4 signaling is required for outflow-tract septation and branchial-arch artery remodeling. Proc. Natl. Acad. Sci. 101: 4489–4494.
Leffredo, C.A., Wilson, P.D., and Ferenčec, C. 2001. Maternal diabetes: An independent risk factor for major cardiovascular malformations with increased mortality of affected infants. Teratology 64: 98–106.
Lynch, S.A., Wang, Y., Strachan, T., Burn, J., and Lindsay, S. 2000. Autosomal dominant sacral agenesis: Currarino syndrome. J. Med. Genet. 37: 561–566.
McPherron, A.C., Lawler, A.M., and Lee, S.J. 1999. Regulation of anterior/posterior patterning of the axial skeleton by growth/differentiation factor 11. Nat. Genet. 22: 260–264.
Menno, C., Shimono, A., Saijoh, Y., Yashiro, K., Mochida, K., Ohishi, S., Noji, S., Kondoh, H., and Hamada, H. 1998. Lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. Cell 94: 287–297.
Mesnard, D., Guzman-Ayala, M., and Constam, D.B. 2006. Nodal specifies embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning. Development 133: 2497–2505.
Morris, A.P. 2006. A flexible Bayesian framework for modeling haplotype association with disease, allowing for dominance effects of the underlying causative variants. Am. J. Hum. Genet. 79: 679–694.
Nakashima, M., Toyono, T., Akamine, A., and Joyner, A.L. 1999. Expression of growth/differentiation factor 11, a new member of the BMP/TGFβ superfamily during mouse embryogenesis. Mech. Dev. 80: 185–189.
Oh, S.P. and Li, E. 1997. The signaling pathway mediated by the type IIb activin receptor controls axial patterning and lateral asymmetry in the mouse. Genes & Dev. 11: 1812–1826.
Oh, S.P., Yeo, C.Y., Lee, Y., Schrewé, H., Whitman, M., and Li, E. 2002. Activin type IIA and IIB receptors mediate Gdf11 signaling in axial vertebral patterning. Genes & Dev. 16: 2749–2754.
Ovchinnikov, D.A., Selever, J., Wang, Y., Chen, Y.T., Mishina, Y., Martin, J.F., and Behringer, R.R. 2006. BMP receptor type IA in limb bud mesenchyme regulates distal outgrowth and patterning. Dev. Biol. 295: 103–115.
Papathanasiou, P. and Goodnow, C.C. 2005. Connecting mammalian genome with phenotype by ENU mouse mutagenesis: Gene combinations specifying the immune system. Annu. Rev. Genet. 39: 241–262.
Petrini, J., Dumas, K., Russell, R., Poschmann, K., Davidoff, M.J., and Mattison, D. 2002. Contribution of birth defects to infant mortality in the United States. Teratology 66 (Suppl. 1): S3–S6. doi: 10.1002/tera.90002.
Rancourt, S.L. and Rancourt, D.E. 1997. Murine subtilisin-like protease SPC6 is expressed during embryonic implantation, somitogenesis, and skeletal formation. Dev. Genet. 21: 75–81.
Ringrose, L. and Paro, R. 2007. Polycomb/Trithorax response elements and epigenetic memory of cell identity. Development 134: 223–232.
Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., et al. 2007. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 129: 1311–1323.
Roebroek, A.J., Umano, L., Pauli, I.G., Robertson, E.J., van Leuven, F., Van de Ven, W.J., and Constam, D.B. 1998. Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. Development 125: 4863–4876.
Ross, A.J., Ruiz-Perez, V., Wang, Y., Hagan, D.M., Scherer, S., Lynch, S.A., Lindsay, S., Custard, E., Belloni, E., Wilson, D.L., et al. 1998. A homeobox gene, HLXB9, is the major locus for dominantly inherited sacral agenesis. Nat. Genet. 20: 358–361.
Schneider, J.E., Bose, J., Bamforth, S.D., Gruber, A.D., Broadbent, C., Clarke, K., Neubauer, S., Lengeling, A., and Bhattacharya, S. 2004. Identification of cardiac malformations in mice lacking Ptdsr using a novel high-throughput magnetic resonance imaging technique. BMC Dev. Biol. 4: 16. doi: 10.1186/1471-213X-4-16.
Seidah, N.G. and Chretien, M. 1999. Proprotein and prohormone convertases: A family of subtilases generating diverse bioactive polypeptides. Brain Res. 848: 45–62.
Sessa, L., Breiling, A., Lavorgna, G., Silvestri, L., Casari, G., and Orlando, V. 2007. Noncoding RNA synthesis and loss of Polycomb group repression accompanies the colinear activation of the human HOXA cluster. RNA 13: 223–239.
Showell, C., Binder, O., and Conlon, F.L. 2004. T-box genes in early embryogenesis. Dev. Dyn. 229: 201–218.
Ueda, K., Lipkind, G.M., Zhou, A., Zhu, X., Kuznetsov, A., Philipson, L., Gardner, P., Zhang, C., and Steiner, D.F. 2003. Mutational analysis of predicted interactions between the catalytic and P domains of prohormone convertase 3 [PC3/PC1]. Proc. Natl. Acad. Sci. 100: 5622–5627.
Ulloa, L., Creemers, J.W., Roy, S., Li, S., Mason, J., and Tabibzadeh, S. 2001. Lefty proteins exhibit unique processing and activate the MAPK pathway. J. Biol. Chem. 276: 21387–21396.
Walder, R.Y., Landau, D., Meyer, P., Shalev, H., Tsolia, M., Borowochitz, Z., Boettger, M.B., Beck, G.E., Englehardt, R.K., Carmi, R., et al. 2002. Mutation of TRPM6 causes familial hypomagnesemia with secondary hypocalcemia. Nat. Genet. 31: 171–174.
Wilkik, D.M. and Capecchi, M.R. 2003. Hox11 and Hox11 genes are required to globally pattern the mammalian skeleton. Science 301: 363–367.
Wilkik, D.M., Hawkes, P.J., and Capecchi, M.R. 2002. Hox11 paralogous genes are essential for metanephric kidney induction. Genes & Dev. 16: 1423–1432.
Xiang, Y., Molloy, S.S., Thomas, L., and Thomas, G. 2000. The PC6b cytoplasmic domain contains two acidic clusters that direct sorting to distinct trans-Golgi network/endoosomal compartments. Mol. Biol. Cell 11: 1257–1273.
Yan, Y.T., Liu, J.J., Luo, Y.J., Lau, Y.E.C., Haltiwanger, R.S., Abate-Shen, C., and Shen, M.M. 2002. Dual roles of Cripto as a ligand and coreceptor in the nodal signaling pathway. Mol. Cell. Biol. 22: 4439–4449.
Yu, Q., Shen, Y., Chatterjee, B., Siegfried, B.H., Leatherbury, L., Rosenthal, J., Lucas, J.F., Wessels, A., Spurrey, C.F., Wu, Y.J., et al. 2004. ENU induced mutations causing congenital cardiovascular anomalies. Development 131: 6211–6223.
Zhou, A., Martin, S., Lipkind, G., LaMendola, J., and Steiner, D.F. 1998. Regulatory roles of the P domain of the subtilisin-like prohormone convertases. J. Biol. Chem. 273: 11107–11114.
VACTERL/caudal regression/Currarino syndrome-like malformations in mice with mutation in the proprotein convertase $Pcsk5$

Dorota Szumska, Guido Pieles, Rachid Essalmani, et al.

*Genes Dev.* 2008, 22: Access the most recent version at doi:10.1101/gad.479408

**Supplemental Material**

http://genesdev.cshlp.org/content/suppl/2008/05/21/22.11.1465.DC1

**References**

This article cites 63 articles, 31 of which can be accessed free at: http://genesdev.cshlp.org/content/22/11/1465.full.html#ref-list-1

**License**

Freely available online through the Genes & Development Open Access option.

**Email Alerting Service**

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.