Crim1– a regulator of developmental organogenesis.

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Abstract

The regulation of growth factor localization, availability and activity is critical during embryogenesis to ensure appropriate organogenesis. This process is regulated through the coordinated expression of growth factors and their cognate receptors, as well as via proteins that can bind, sequester or localize growth factors to distinct locations. One such protein is the transmembrane protein Crim1. This protein has been shown to be expressed broadly within the developing embryo, and to regulate organogenesis within the eye, kidney and placenta. Mechanistically, Crim1 has been revealed to mediate organogenesis via its interaction with growth factors including TGFβs, BMPs, VEGFs and PDFGs. More recently, Crim1 has been shown to influence cardiac development, providing further insights into the function of this protein. This review will provide an overview of the role of Crim1 in organogenesis, largely focusing on how this protein regulates growth factor signaling in the nascent heart. Moreover, we will address the challenges ahead relating to further elucidating how Crim1 functions during development.

List of Abbreviations

BMP – Bone Morphogenetic Protein

Crim1 – Cysteine-Rich Transmembrane BMP Regulator-1

CRR – Cysteine-rich Repeat

EMT – Epithelial-to-Mesenchymal Transition

EndoMT – Endothelial-to-Mesenchymal Transition

EPDC – Epicardium-Derived Cells

ERK – Extracellular signal-Regulated Kinase

FAK – Focal Adhesion Kinase

Gbb – Glass bottom boat

HUVEC – Human Umbilical Vein Endothelial Cell

IGF – Insulin-like Growth Factor

IGFBP – Insulin-like Growth Factor Binding Protein

NMJ – Neuromuscular Junction

PDGF – Platelet-Derived Growth Factor

PE – Proepicardium

RGD – Arginyl-glycyl-aspartic acid motif

TGFβ – Transforming Growth Factor β

VEGF – Vascular Endothelial Growth Factor

Introduction

Cysteine-Rich Transmembrane BMP Regulator-1 (Crim1) is a novel, *N*-glycosylated transmembrane protein encoded by the *Crim1* gene (Kolle *et al.*, 2000; Glienke *et al.*, 2002). *Crim1* genes are evolutionarily conserved in vertebrates including rodents and humans (Kolle *et al.*, 2000), as well as zebrafish (Kinna *et al.*, 2006), the chicken (Kolle *et al.*, 2003) and *Xenopus* (Ponferrada *et al.*, 2012). Interestingly, a Crim1 homolog called crm-1 has been described in *Caenorhabditis elegans* as well, although the role of this factor in the nematode has only received limited attention (Fung *et al.*, 2007). Crim1 is expressed in various organs during embryogenesis, including the spinal cord, lens, kidney, vasculature and placenta (Kolle *et al.*, 2000; Lovicu *et al.*, 2000; Glienke *et al.*, 2002; Kolle *et al.*, 2003; Pennisi *et al.*, 2007; Pennisi *et al.*, 2012; Phua *et al.*, 2012; Fan *et al.*, 2014), indicative of a role in their development. Structurally, the presence of six cysteine-rich repeat (CRR) motifs, an Insulin-like Growth Factor (IGF) Binding Protein (IGFBP) like domain and an Arginyl-glycyl-aspartic acid motif (RGD) (Kolle *et al.*, 2000) suggests that Crim1 can bind a variety of different proteins. In support of this, the CRRs have been shown to mediate the binding of CRIM1 to TGFβ, BMP, VEGF and PDGF when Crim1 is co-expressed in the same cell as the growth factor (Wilkinson *et al.*, 2003; Wilkinson *et al.*, 2007). Recently, Crim1 has also been shown to interact with β1 Integrin via its RGD domain (Zhang *et al.*, 2016). However, the functional significance of the IGFBP motif remains unknown. Interestingly, Crim1 has been demonstrated to localize in the endoplasmic reticulum and golgi, where post translational modification of proteins are known to occur, and also the cell surface, where ligand-receptor interactions occur (Glienke *et al.*, 2002; Wilkinson *et al.*, 2003), Pennisi lab, unpublished data).

The importance of proteins that regulate growth factor signalling has been highlighted by recent findings that implicate molecules containing multiple cysteine-rich regions, such as chordin and noggin, during development. For example, BMP signal transduction is regulated by various proteins that exert either a pro- or anti-BMP effect. Mediation of this pathway can occur at multiple levels, such as by sequestering BMPs and either facilitating or inhibiting BMP-receptor interactions, hindering BMP transport across cells and thus disturbing the BMP gradient required for downstream signaling, or by maintaining some BMPs in inactive form by preventing the cleavage of pre-BMPs and reducing the amount of mature, secreted

BMPs (Wilkinson *et al.*, 2003; Umulis *et al.*, 2009). Chordin is an antagonist that directly binds BMPs (Larraín *et al.*, 2000), and noggin, a cysteine-knot protein that binds BMPs and prevents them from binding to type I and type II cell surface BMPRs via masking of receptor binding sites (Zimmerman *et al.*, 1996; Groppe *et al.*, 2002). On the other hand, crossveinless-2 potentiates BMP signaling by forming a ternary complex with both chordin and BMP, and reduces the affinity of chordin for BMP, thus allowing BMPs to activate their downstream effectors via the BMPRs (Zhang *et al.*, 2010). Importantly, the action of these proteins is dependent of the developmental context in which they, and the molecules they interact with, are expressed. This has been highlighted by *Crim1*-deficient mice, which exhibit phenotypes that indicate Crim1 can perform both agonistic and antagonistic functions on growth factor signaling during organogenesis.

To explore the role of Crim1 in development, a number of transgenic mice have been generated. Firstly, a genetrap line was created by random insertion of a β-geo cassette into intron 1 of the *Crim1* gene (called $Crim1^{KST264}$) (Leighton *et al.*, 2001; Pennisi *et al.*, 2007). Secondly, a conditional loss-of-function allele was generated ($Crim1^{FLOX}$), by flanking exons 3 and 4 of the *Crim1* gene with LoxP sites (Chiu *et al.*, 2012). The $Crim1^{FLOX}$ line, when crossed with the *CMV-Cre* deletor line, generated $Crim1^{\Delta flox}$ mice, intercrosses from which produced mice lacking a functional *Crim1* gene (called $Crim1^{\Delta flox/\Delta flox}$) (Chiu *et al.*, 2012). We have previously described perinatal lethality in mice homozygous for the $Crim1^{KST264}$ genetrap and in $Crim1^{\Delta flox/\Delta flox}$ mice (Pennisi *et al.*, 2007; Chiu *et al.*, 2012). Both strains display defects in multiple organ systems including the kidney, eye and placenta (Lovicu *et al.*, 2000; Glienke *et al.*, 2002; Pennisi *et al.*, 2007; Pennisi *et al.*, 2012; Phua *et al.*, 2012; Fan *et al.*, 2014), highlighting the importance of Crim1 during development. In this review we discuss the role of Crim1 in organogenesis, with a specific focus on the developing heart, as well as providing mechanistic insight into how it can regulate the activity of growth factors.

The role of Crim1 in organogenesis

In the past 15 years a number of studies have begun to map the expression of Crim1 within the developing embryo, and have used the different transgenic mouse lines described above to decipher the role for Crim1 in organogenesis. A summary of these findings is detailed below.

Kidney

Perhaps the most widely studied organ in the context of Crim1 function is the kidney. In the embryonic murine kidney, Crim1 is expressed in pericytes lining the endothelium and within the parietal epithelial cells, mesangial cells and podocytes of the glomeruli from 15.5 days post coitum (dpc) (Georgas *et al.*, 2000; Pennisi *et al.*, 2007; Wilkinson *et al.*, 2007). Studies of transgenic animals have provided significant insights into the role of Crim1 during kidney development. Kidneys from $Crim1^{KST264/KST264}$ mice at 15.5 dpc were significantly smaller than their wild type littermates, indicating a role for Crim1 in nephrogenesis (Pennisi *et al.*, 2007). Further analysis revealed multiple lesions in the glomerulus and glomerular capillary defects, and podocyte effacement (Wilkinson *et al.*, 2007). In their elegant study, Wilkinson et al. showed Crim1 to be co-expressed with VEGF-A in the podocytes of the renal glomerulus. In mice lacking *Crim1*, there was an increased diffusion of VEGF-A away from the podocytes at 17.5 dpc, and a concomitant activation of the VEGF-A receptor Flk1 in adjacent vascular endothelial cells, supporting the observation of glomerular defects in these homozygotic mice (Wilkinson *et al.*, 2007). To assess whether renal abnormalities were present in the adult kidney, Wilkinson et al. made use of $Crim1^{KST264/KST264}$ outbred mice, as a proportion of these homozygous animals survive to adulthood (Wilkinson *et al.*, 2007). The kidneys of these adult mice displayed multiple glomerular cysts, interstitial fibrosis and endothelial cell thickening (Wilkinson *et al.*, 2007), accompanied by further evidence of increased vascular leakiness and compromised extraglomerular vasculature (Wilkinson *et al.*, 2009). Furthermore, a later study revealed renal fibrosis in $Crim1^{KST264/KST264}$ adult mice along with endothelial aberrations, including an increase in vascular permeability and a discontinuous endothelium displaying abnormal collagen deposits (Phua *et al.*, 2012). This could be due to the association of Crim1 with TGFβ-1, which is known stimulate endothelial-to-mesenchymal transformation (EndoMT) (Kim *et al.*, 2001; Varga and Wrana, 2005; Phua *et al.*, 2012). In the adult human kidney, CRIM1 has been shown to be localized in the podocytes, both qualitatively, using renal tissue in immunohistochemical experiments, and quantitatively, using immortalised human podocytes (Nyström *et al.*, 2009), where BMPs and VEGFs are also expressed (Simon *et al.*, 1995; Godin *et al.*, 1999), indicating a possible role for CRIM1 in tethering these growth factors and releasing them into the local environment in a controlled manner.

Placenta

The placenta plays a central role during embryogenesis. It comprises a labyrinthine zone consisting of syncytiotrophoblast cells and junctional zone consisting of spongiotrophoblast and glycogen trophoblast cells. In mice, Crim1 is expressed in various placental cell types including chorionic trophoblasts at 9.5 dpc, synctiotrophoblasts at 13.5 and 15.5 dpc, and spongiotrophoblasts from 13.5 dpc (Pennisi *et al.*, 2012). *Crim1* is important for placental development as, in the absence of this gene, placental size is reduced from 13.5 dpc until 17.5 dpc, with a consequent reduction in the size of $Crim1^{KST264/KST264}$ embryos at the later stage (Pennisi *et al.*, 2012). There is also a decrease in the number of sinusoidal-trophoblast giant cells and an increase in glycogen cells of $Crim1^{KST264/KST264}$ placentae at 15.5 dpc, hypothesized to be due to a possible dysregulation of multiple signalling pathways (Pennisi *et al.*, 2012), such as the IGF and VEGF pathways (Charnock-Jones *et al.*, 1994; Randhawa and Cohen, 2005).

Lens and retinal vasculature

In the developing murine embryonic eye, *Crim1* expression is observed from 9.5 dpc until at least day postnatal day (P) 21 (Lovicu *et al.*, 2000). Initially, *Crim1* is detected in the precursor to the lens, the lens placode, and subsequently is expressed by all lens cells by 11.5 dpc. *Crim1* transcripts are also present in the corneal epithelium and endothelium by 15.5 dpc, as well as the retinal epithelium and retinal ganglion cells at 18.5 dpc. At P21, *Crim1* expression persists only in the lens (Lovicu *et al.*, 2000). Analysis of $Crim1^{glcr11}$ *(glaucoma relevant 11)* mutants, termed so because of their glaucoma and cataract phenotype, reveals multiple lens defects that are evident from 16.5 dpc, including abnormal cell adhesion at epithelial adhesion junctions, disrupted polarity and a reduction in the number of proliferating lens epithelial cells, which collectively culminate in a smaller, atypical lens (Zhang *et al.*, 2016). By P60, *Crim1* mutant mice also display lens cataracts and abnormal cellular proliferation within the retina. Mechanistically, the adhesion defects in these *Crim1* mutants are consistent with an interaction observed between Crim1 and β1 integrin, which is also expressed in both lens epithelial cells and lens fiber cells (Zhang *et al.*, 2016). Indeed, analysis of β1 integrin signalling reveals that Crim1 regulates the phosphorylation status of its downstream effectors focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK), resulting in modulation of lens morphogenesis by membrane-bound Crim1 (Zhang *et al.*, 2016).

As Crim1 is expressed *in vivo* and *in vitro* in vascular endothelial cells (Glienke *et al.*, 2002), and in the vasculature of the embryonic mouse hindbrain and postnatal retinas (Fan *et al.*, 2014), its role in the retinal vasculature has also been analysed. Crossing the $Crim1^{Flox}$ allele to *Pdgfrb-iCreER* mice to enable inducible deletion of *Crim1* from endothelial cells reveals that defective retinal vascular development occurs in the absence of *Crim1* from the vasculature (Fan *et al.*, 2014). The phenotypes observed included reduced vessel density, length and branchpoint number, and vessel regression in the first week of postnatal development (Fan *et al.*, 2014). Indeed, these authors revealed modulation of the autocrine activity of VEGF-A by Crim1, indicating that it has an important regulatory role in the formation and development of the vasculature (Fan *et al.*, 2014). The distribution of the cell adhesion molecule VE-Cadherin at the angiogenic front of $Crim1^{flox/flox}$*; Pdgfb-iCreER* retinal vasculature preparations was also altered (Fan *et al.*, 2014), a finding consistent with the impaired endothelial tube formation evident in HUVECs in the absence of *CRIM1* (Glienke *et al.*, 2002). This is suggestive of a cell adhesion anomaly. A possible role for the conserved RGD motif in Crim1 in this context is possible, as this domain of Crim1 potentially binds integrins and so modulates cellular attachment (Kolle *et al.*, 2000). The intracellular domain of Crim1 could also play a role in cell adhesion, as the cytoplasmic domain of Crim1 has been shown to indirectly bind β-catenin and N-cadherin in *Xenopus* (Ponferrada *et al.*, 2012).

Nervous system

Preliminary investigations have also shown that Crim1 may be important for the development of the nervous system. For instance, in the developing mouse spinal cord, Crim1 is expressed from 9.5 dpc in the floor plate, and in pools of motor neurons at later stages of development (Kolle *et al.*, 2000). Moreover, Crim1 expression is observed in other regions of the nascent mouse neuraxis, including the forebrain and hindbrain from 11.5 dpc, and in the midbrain at 13.5dpc, (Kolle *et al.*, 2000). Despite this, studies have yet to elucidate the mechanistic function of Crim1 in the developing nervous system. Looking forwards, using our understanding of the role of Crim1 in other developmental contexts may provide insights into the role of this factor within the developing nervous system. For example, the colocalization of Crim1 and β1 integrins at the leading edges of lens epithelial cell projections regulates cell adhesion and polarity (Zhang *et al.*, 2016). As cellular adhesion and polarity are critical components that underlie the proliferation and subsequent differentiation of neural stem cells

within the embryonic brain, a role for Crim1 in mediating these aspects during neural development is plausible.

The role of Crim1 in cardiac development

Overview of organogenesis of the heart

Another organ in which Crim1 plays an important role during development is the heart. The heart is the first organ to form and function in the vertebrate embryo (Yutzey and Kirby, 2002), and cardiac progenitor cells are among the earliest to migrate through the primitive streak during gastrulation (Garcia-Martinez and Schoenwolf, 1993; Schoenwolf and Garcia-Martinez, 1995). Splanchnic mesenchymal cells, the principal cardiac precursors, arise from primary heart fields in the lateral plate mesoderm (Waldo *et al.*, 2001) and aggregate in the cardiogenic region to form angioblastic cords. These canalize to form two thin-walled endocardial heart tubes. Their subsequent fusion forms a single two-layered heart tube (Manasek, 1969) comprising the outer mesenchymal myocardial mantle which forms the myocardium, and the inner endothelial tube which forms the endocardium, separated by myocardium-produced cardiac jelly (Waldo *et al.*, 1999).

The epicardium of the heart develops from a transient structure, the proepicardium (PE) (Virágh and Challice, 1981; Männer, 1993; Virágh *et al.*, 1993; Gittenberger-de Groot *et al.*, 1998), that is located between the sinus horns and liver primordium (Virágh *et al.*, 1993), and which is derived from the lateral plate mesoderm (Serluca, 2008). The PE contains smooth muscle, fibroblast and endothelial progenitors (Mikawa and Gourdie, 1996; Dettman *et al.*, 1998; Gittenberger-de Groot *et al.*, 1998; Männer, 1999; Pérez-Pomares *et al.*, 2002), but whether it is the sole contributor of endothelial cells to the coronary vasculature is contentious, as the liver bud and sinus venosus have also been suggested as sources of the same (Poelmann *et al.*, 1993; Ishii *et al.*, 2007; Red-Horse *et al.*, 2010; Cossette and Misra, 2011).

Proepicardial cells attach to the inner curvature of the atrioventricular junction of the rudimentary heart (Männer, 1993; Ishii *et al.*, 2010) to form its outermost layer – the epicardium. Species-specific migration of proepicardial cells to the myocardium occurs either by proepicardial vesicle budding or via an extracellular matrix bridge to potentially guide the translocation (Nahirney *et al.*, 2003). It has been suggested that the proximity between the liver bud and the PE affects proepicardial attachment to the heart and differentiation via an

associated effect on proepicardial marker genes (Ishii *et al.*, 2007). Following the attachment, a population of epicardial cells undergo EMT to form subepicardial cells, including those contributing to the subepicardial coronary vasculature, whereas another subset of these cells traverse the subepicardial space and migrate into the myocardium (Virágh and Challice, 1981) to give rise to various cell types, including coronary vascular smooth muscle cells, coronary vascular endothelial cells and cardiac fibroblasts (Virágh and Challice, 1981; Männer, 1993; Virágh *et al.*, 1993; Mikawa and Gourdie, 1996; Gittenberger-de Groot *et al.*, 1998).

Crim1 and cardiac development

What evidence is there that Crim1 plays a role in cardiac development? Firstly, studies on the *Crim1*^{KST264} genetrap line (which carries a LacZ reporter), have shown that *Crim1*-promoter mediated LacZ expression is evident in the murine proepicardium at 9.5 dpc, and within the epicardium throughout cardiac development. It is also observed in coronary vascular smooth muscle cells and, to a weaker extent, in coronary vascular endothelial cells, at later stages of heart development (Pennisi *et al.*, 2007). LacZ expression is also observed in the outflow tract mesenchyme, bicuspid and tricuspid valve leaflets and atrial septum at 18.5 dpc (Iyer *et al.*, 2016). These sites of expression imply that Crim1 may regulate many aspects of cardiac development. Interestingly, there are many cardiac phenotypes, such as chamber septation and valve defects, hypoplastic ventricular walls and coronary vasculature defects, that arise as a result of dysregulation of growth factors such including TGFβs, BMPs, VEGFs and IGFs (Kim *et al.*, 2001; Chen *et al.*, 2004; Goldman *et al.*, 2009; Uchimura *et al.*, 2009; Li *et al.*, 2011; Wu *et al.*, 2012). This indicates that growth factor activity is normally tightly controlled during cardiogenesis. Given the interaction of Crim1 with many of these factors in other organ systems, and preliminary data indicating that *Crim1* mutant cardiac phenotypes are reminiscent of these phenotypes, we posit that Crim1 mediates cardiogenesis, at least in part, via the regulation of growth factor signalling.

For example, epicardial EMT is a vital process that occurs during normal heart development, and several growth factors have been implicated in both EMT, and the differentiation of epicardial cells into their correct lineages. A number of distinct molecular processes work cooperatively in order to initiate and promote epicardial EMT. As the heart develops, there is significant cross-talk between the epicardium and myocardium, with epicardial signalling via its secreted factors and epicardium-derived cells, as well as signalling from the myocardium,

being essential for myocardial growth and differentiation (Sucov *et al.*, 2009) and coronary vascular development (Kang and Sucov, 2005). Thus, it is this reciprocal signalling between the epicardium and myocardium which provides cues to ensure the proper and timely differentiation of epicardial lineages, maturation of the myocardium and the coronary vasculature, and, ultimately, the development and functioning of the heart. For instance, TGFβs stimulate epicardial EMT (Dokic and Dettman, 2006; Olivey *et al.*, 2006; Sánchez and Barnett, 2012). Binding of TGFβ2 and TGFβ3 to TGFβR2, and the subsequent activation of TGFβR1, leads to the phosphorylation of SMAD2/3 proteins and upregulation of transcription factors such as snail1 and slug. These factors repress the expression of E-cadherin, while promoting the expression of Vimentin, RhoA and various ECM molecules (Xu *et al.*, 2009), thus facilitating a transition away from epithelial characteristics. Using the *WT1-Cre* and *WT1-CreERT2* cell lines, we have shown that EMT and epicardial migration are increased in the absence of *Crim1* (Iyer *et al.*, 2016). The epicardium of *Crim1* null mutant hearts surprisingly shows a reduced phospho-SMAD2 level, indicative of reduced TGFβ signalling (Iyer *et al.*, 2016), at 13.5 dpc, despite enhanced EMT. This indicates that there could be a role for Crim1 in the formation or stabilization of cadherin-dependent junctional complexes in epithelial cells, via which it could serve to normally restrain epicardial EMT. β-catenin is a crucial component of adherens junctions, and has been previously shown to complex indirectly with Crim1 (Ponferrada *et al.*, 2012). Indeed, assessment of β-Catenin distribution at epicardial cell-cell junctions is altered in *Crim1*-deficient mice, indicating a loss of stability at these contact points within the developing heart (Iyer *et al.*, 2016).

Epithelial and mesenchymal cells secrete PDGF-A, which may act as a mitogen that stimulates ventricular development and cardiomyocyte proliferation (Kang *et al.*, 2008). Moreover, PDGF-B can stimulate proepicardial cells expressing smooth muscle markers to undergo epicardial EMT and subsequently commit to the coronary smooth muscle cell lineage, mediated through PDGFR-β (Lu *et al.*, 2001), while PDGFR-α plays an important role in the formation of cardiac fibroblasts (Smith *et al.*, 2011). Both PDGF receptors are implicated in EMT, whereby epicardium-derived cells (EPDCs) give rise to myocardial fibroblasts and vascular smooth muscle cells (Mellgren *et al.*, 2008; Smith *et al.*, 2011). We have recently reported a reduction in the number of EPDC-derived myocardial fibroblasts within *Crim1* mutant mice (Iyer *et al.*, 2016). Although Crim1 has been shown to capable of binding PDGF-B (Wilkinson *et al.*, 2007), direct evidence to support a role of Crim1 in the

modulation of this pathway in the heart is currently lacking. Future work aimed at investigating this exciting prospect will undoubtedly advance our understanding of the mechanism through which Crim1 regulates the biology of EPDC-derived cells. Moreover, PDGF-B is also expressed by endothelial cells is required for the endothelial-cell-mediated recruitment for coronary vascular smooth muscle cells to the developing coronary vessels (Van den Akker *et al.*, 2008). Given the nature of the Crim1 protein, this cysteine-knot protein could also potentially antagonise this aspect of cardiac development by tethering PDGFs to the cell surface and limiting their action, another fruitful avenue of future research.

Interestingly, in support of this hypothesis, Wilkinson et al. previously identified that Crim1 potentially functions as an antagonistic regulator of certain members of the BMP family. Crim1 interacts intracellularly with both BMP4 and BMP7 (Wilkinson *et al.*, 2003), and co-localizes with their respective pre-BMPs within the golgi via its CRRs, ultimately reducing the secretion of mature BMPs. Furthermore, a proportion of the BMPs released remain bound to Crim1 (Wilkinson *et al.*, 2003). Crim1 has also been implicated in tethering BMPs to the cell, which may serve to restrict their functional potential, and, since BMPs are known to act across a restricted distance (Jones *et al.*, 1996), possibly to act in the presentation of BMP ligands to neighbouring cells. BMPs are well known for their role in cardiac development. For instance, BMP2 increases epicardial EMT via TGFβR3 activation in epicardial cell lines (Sánchez and Barnett, 2012), and BMP4 has been shown to play an important role in both atrioventricular and outflow tract septation (Jiao *et al.*, 2003; Liu *et al.*, 2004). The absence of *Bmp10* leads to impaired ventricular trabeculation and formation of thin ventricular walls (Neuhaus *et al.*, 1999), a phenotype recapitulated in mice lacking both *Bmp* 6 and *Bmp7* (Kim *et al.*, 2001). Given the need for exquisite spatial and temporal modulation if BMP signaling, it is likely that Crim1 also regulates this family of molecules during cardiac development, Indeed, hypoplastic ventricles are observed in *Crim1* null mice, alongside a concomitant increase in apoptosis of intramural cells, indicating that Crim1 is necessary for the formation of the myocardium, potentially via the modulation of BMP signalling (Iyer *et al.*, 2016). The use of next generation sequencing in *Crim1*-deficient mice, coupled with proteomic approaches, could provide a future avenue to determine the role Crim1 plays in the modulation of BMP biology during cardiac development.

IGFs have been also implicated as epicardial mitogenic factors during heart formation. For instance, IGF-2 is secreted from the epicardium and exerts a mitogenic effect on the

formation of the compact myocardium (Li *et al.*, 2011). Could the IGFBP motif of Crim1, along with the CRR domains, bind IGFs and regulate their activity? Interestingly, there is an increase in ERK1/2 signalling in the myocardium of *Crim1*–deficient hearts at 13.5 dpc (Iyer *et al.*, 2016). This indicates that Crim1 regulates signalling molecules secreted by the epicardium, or by the myocardium itself, and is thus essential for myocardial maturation in the early stages of heart development. A large number of growth factors are known to activate the ERK pathway, including IGFs, and downstream ERK signalling can be both pro- and anti-apoptotic, making it important to identify which growth factors are specifically involved in this process, and exactly how this augmented ERK1/2 signalling affects the development of the myocardium. It would thus be useful to assess whether Crim1 can indeed bind IGFs, and further whether Crim1 can modulate this important growth factor in the context of the developing heart.

Concluding remarks

While the broad role of Crim1 in developmental organogenesis is now well established, much remains unclear as to how this transmembrane protein exerts its biological influence. Critically, the mechanisms by which Crim1 mediates growth factor signalling in different developmental contexts are still poorly defined. For example, in *C. elegans*, crm-1 acts as an agonist of a BMP-like pathway, the DBL-1 pathway, in a non-cell-autonomous fashion (Fung *et al.*, 2007), although it is not known whether this occurs through an interaction with the ligand or its receptor. In contrast to this agonistic role with regards to BMP signalling, the *Drosophila* homolog of Crim1, CRIMPY, antagonizes the function of the BMP ligand Glass bottom boat (Gbb) in motorneurons at the neuromuscular junction (NMJ), and restrains the expansion of the NMJ (James and Broihier, 2011). The full-length Gbb precursor associates preferentially with the extracellular domain of CRIMPY to regulate synaptic development, before Gbb is secreted from the motorneuron terminal (James and Broihier, 2011). Similarly, Crim1 binds to and antagonistically modulates the processing of pre-BMPs and the secretion of mature BMPs in COS7 cells (Wilkinson *et al.*, 2003). These findings highlight the fact that the function of Crim1 in organogenesis is context-dependent, and that the timing and site of Crim1 expression, coupled with that of the multitude of growth factors it can potentially interact with, will influence its biological function.

Looking to the future, investigations into Crim1 function during development will enable us to probe the mechanisms underlying a variety of pathological disorders. For instance, the

adult myocardium has limited regenerative capacity. New technologies have made the generation of cardiomyocytes from induced stem cells a possibility, but it remains essential to clarify the growth factors and signalling molecules germane to this process to make this feasible. BMPs, for instance, regulate stem cell renewal and differentiation into cardiomyocytes, and cooperate with other signalling pathways to further modulate gene expression of transcription factors (Varga and Wrana, 2005; van Wijk *et al.*, 2007). As Crim1 can regulate BMP processing, investigating the intersection between Crim1 biology and BMP signalling during the generation of cardiomyocytes *in vitro* could be a valuable approach. Similarly, research on the reactivation of the adult epicardium following myocardial damage has increased in recent years, and the modulation of growth factors such as BMPs, TGFβ, VEGF and PDGF by Crim1 is an attractive avenue of research that remains to be explored. Moreover, as myocardial injury stimulates epicardial cells to give rise to fibroblasts and smooth muscle cells (Limana *et al.*, 2011; Zhou *et al.*, 2011; Duan *et al.*, 2012; Huang *et al.*, 2012; van Wijk *et al.*, 2012), our recent observation that, in the absence of *Crim1*, the number of cardiac fibroblasts is reduced (Iyer *et al.*, 2016), is another step towards deciphering its role not only in lineage specification but also potential therapeutic interventions to improve cardiac performance after damage.

With relation to other pathological disorders, the analysis of serum from chronic heart failure patients shows an increase in CRIM1 levels, along with an increase in secreted factors involved in fibrosis, indicating a positive correlation between CRIM1 and pro-fibrotic activity (Eleuteri *et al.*, 2014), though whether these increases are reflected within cardiac tissue is not clear. Moreover, CRIM1 is expressed at higher levels in drug-resistant leukaemia cells, implicating it as a potential drug resistance marker (Prenkert *et al.*, 2010). The intronic regions of *CRIM1* and *ZEB2* have been demonstrated to be downregulated in breast cancer epithelial cells (Kim *et al.*, 2015), and *CRIM1* has also been suggested to be a target of the Hippo pathway, and to be overexpressed in gastric cancer tissues (Lim *et al.*, 2014). These tantalising vignettes into the role of CRIM1 in cancer biology and disease provide a platform on which to further investigate the role of this gene in pathological conditions. Indeed, studies from development have illustrated that Crim1 may act at the nexus of many critical signalling pathways, and so the manipulation of *Crim1* expression may provide a parsimonious mechanism by which cellular functions such as proliferation, differentiation and repair can be efficiently manipulated following injury and tumorigenesis.

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