ADAM function in embryogenesis

Dominique Alfandari, **Catherine McCusker**, and **Hélène Cousin**
Department of Veterinary and Animal Sciences, University of Massachusetts Amherst., Paige lab. Rm. 203, 161 Holdsworth Way, Amherst, MA 01003

Abstract

Cleavage of proteins inserted into the plasma membrane (shedding) is an essential process controlling many biological functions including cell signaling, cell adhesion and migration as well as proliferation and differentiation. ADAM surface metalloproteases have been shown to play an essential role in these processes. Gene inactivation during embryonic development have provided evidence of the central role of ADAM proteins in nematodes, flies, frogs, birds and mammals. The relative contribution of four subfamilies of ADAM proteins to developmental processes is the focus of this review.

Keywords

1. Introduction

ADAM proteins are single-pass transmembrane metalloproteases that contain a disintegrin domain. They were initially identified as sperm-specific proteins with similarity to snake venom metalloproteases [1-7]. In snakes the disintegrin domain binds with high affinity to the platelet integrins, inhibiting platelet aggregation, while the metalloprotease domain cleaves components of the endothelial basement membrane inducing blood vessel leakage and hemorrhage. During the past 12 years, ADAMs have been identified in a wide variety of animal species including nematodes, sea urchin, fruit flies, frogs, birds and mammals. Detailed analyses of the expression of ADAMs have shown that far from being restricted to spermatozoa, these proteins are expressed in all cell types. While our understanding of ADAM protein function has improved tremendously, it is still a growing field with many questions left unanswered. Many excellent reviews have looked at the various aspects of ADAM biology [8-18].

The basic ADAM structure is represented in Figure 1. All ADAMs contain a prodomain, a metalloprotease domain, a disintegrin and cysteine-rich domain. Most ADAMs also contain an EGF repeat, a transmembrane domain and a cytoplasmic domain. The pro-domain is thought to be essential for protein folding and to prevent the metalloprotease activity [19]. The metalloprotease domain is present even in ADAMs that do not have proteolytic activity. The activity of the metalloprotease domain can be predicted by the presence or absence of the consensus catalytic active site (**HEXGHXXGXXHD**). The substrates for ADAM

Corresponding Author: D. Alfandari. email E-mail: alfandar@vasci.umass.edu, Tel: (413) 577-4269, Fax: (413) 545-6326.

metalloproteases include, cell adhesion molecules, proteins of the extracellular matrix, growth factors and cytokines, as well as receptors and ligands of signaling molecules (Figure 2).

The study of ADAM proteins has shown that many cell surface proteins are indeed cleaved from the cell surface in a process called shedding [20]. This can either activate (Notch or EGF ligands) or inactivate the cleaved proteins (ephrin, cadherins). The disintegrin and cysteine-rich domain of ADAMs have been shown to interact with cell adhesion molecules including the receptors of the extracellular matrix, integrins [21], as well as proteoglycans (e.g. syndecans) [22]. Studies of ephrin cleavage have shown that the cysteine-rich domain also interacts with the substrate and could be responsible for ADAM specificity [23]. The cytoplasmic domain of ADAMs is the most variable part of the molecule and, in some ADAMs, controls the function and the subcellular localization.

In this review we will focus on ADAM function during embryo development using data generated by the powerful genetics in nematodes, fly and mouse, together with results from gain-of- and loss-of-function experiments both in the bird and in frog embryos. Given the large number of potential substrates for each ADAM (Figure 2), we will only discuss those with direct links to the observed phenotypes. Since many reviews have already addressed the role of ADAMs during fertilization, we will focus on later aspects of embryonic development. We will describe the functional evidence by subsets of ADAMs across all species to try to find common trends. The subgroup of ADAMs discussed will be the following: (1) the two main sheddases; ADAM10 and 17, (2) the meltrins; ADAM9, 12, 13/33, and 19, and (3) the ADAMs that have a non-functional metalloprotease domain; ADAM11, 22, 23, and Unc-71. While these groups may appear at first as an artificial distribution, they in fact represent the different ADAMs found in the most primitive organisms such as the nematode, *C. elegans*, in which at least one member of each subgroup is found. Because of space limitations, we will not discuss the role of secreted ADAM-TS despite their essential function during embryogenesis reviewed in [24,25].

2. How to study ADAM function in embryos

The study of ADAM metalloproteases during embryo development provides some specific challenges that may be addressed differently depending on the model chosen. Clearly, the first and most efficient approach has been the loss-of-function experiments. These have been achieved using genetics to inactivate selected genes in the fruit fly, the nematode and the mouse. While this is an extremely powerful approach, it sometimes prevents the study of tissue-specific phenotypes if the overall effect on embryo development is too severe. Loss-of-function studies have also been performed in other vertebrates, such as frog and chick embryos, using Morpholino antisense oligonucleotides that prevent translation of specific mRNA(s) (Figure 3). These can either be injected (in frogs) or electroporated (in chickens). The last approach is the use of Dominant Negative (DN) constructs whose protein product can interfere with the function of an ADAM. This can be achieved by using an ADAM lacking the metalloprotease domain or having a single point mutation in the active proteolytic site, as was shown for ADAM10 and ADAM13 [26,27]. The DN approach was efficiently used for ADAM10 in *Drosophila* to selectively target different tissues and different stages of development (see below). The study of mosaic embryos in *Drosophila* whereby cells lacking ADAMs are placed in embryos possessing the wild type protein, have contributed immensely to our understanding of the ADAM function. In addition, at least one study in mice has tested the ability of wild type ADAM19 to rescue heart defects by expressing the protein in different cell types (described below [28]). This type of study, together with conditional knock outs, will be essential to understanding the function of ADAMs in vertebrate embryos.

While this is the first step of any functional study, in the case of ADAM metalloproteases, the identification of the substrates must follow and is, in most cases, the most challenging part. This is due to the lack of specific cleavage sequences, the large overlap in substrate specificity (Figure 2), and either the absence of phenotype (for most ADAMs) or the multitude of phenotypes (as for ADAM10). This has been addressed very differently in vertebrates and invertebrates. In *Drosophila* and *C.elegans*, genetic complementation has been used to identify target genes and has proven very effective at identifying the various components of the Notch pathway affected by ADAM10. Support for other substrates has not yet been clearly identified using this approach. On the other hand, studies using mammalian cell culture have identified a large number of substrates for each ADAM, defined the overlap and, in some cases the cleavage site, without a direct link to the physiology. By comparing the ability of cells isolated either from wild-type or KO mice to shed the various substrates, the physiological role of each cleavage can be established and, in some cases, linked to the phenotype. In frogs, similar approaches have been undertaken using candidate genes from cell-based assays. In order to confirm the importance of each substrate, rescue experiments introducing the artificially cleaved product of the putative substrate have been initiated (Figure 3). Clearly, the combination of all approaches will be necessary to identify the function and substrates of each ADAM during embryo development.

3. ADAM10 and ADAM17 are the main sheddases

ADAM10 and ADAM17 are the best-characterized ADAMs thus far and appear to have the most important function during embryo development. Mutation of either ADAM10, or ADAM17 produces dramatic phenotypes during early embryogenesis both in vertebrates and invertebrates (see below). Surprisingly, both *Drosophila* and the frog, *Xenopus tropicalis*, have an additional duplication of ADAM10. In *Drosophila* Kuzbanian-like (Kul) function appears critical for a subset of Notch signaling activity. The Notch/Delta signaling pathway is one of the most important during early development and is used primarily when cells are faced with a binary decision: one cell can become or not become a specific cell type. The best example of this has been shown during *Drosophila* neurogenesis when a uniform population of cells needs to decide whether to become neural or epidermal cells. The first cell that expresses an excess of Notch (compared to its neighbors) will become neural and signal to all the cells that are in direct contact that they can't become neural. These cells will up regulate Delta and down regulate Notch, amplifying the initial difference and stabilizing the cell fate decision [29-31].

3.1. ADAM10 role in early cell fate decision

ADAM10 loss-of-function mutation was first identified in *Drosophila* as a mutant with neurogenic phenotype [32]. In these flies, the neural tissue was abnormally large at the expense of the epidermis. Using analyses of mosaic embryos, in which a subset of cells are mutant in a wild-type background, Rooke and colleagues further showed that Kuzbanian was required in two separate events. First, in ectodermal cells to receive the signal that prevents them from adopting the neural fate (lateral inhibition) and second, in cells to generate a signal to promote neuronal fate. They went on to clone the Kuzbanian gene and found that it was homologous to a previously known metalloprotease cloned in bovine and known to cleave the myelin basic protein [33]. This metalloprotease is now known as ADAM10. The same year, Fambrough and colleagues found that in zygotic Kuzbanian-null mutants, axon extension was severely impaired. This was not simply due to the earlier defect in neurogenesis, as some neurons were not affected by the mutation [34]. To be able to turn off the function of ADAM10 in embryos, Pan and colleagues produced a "putative" DN form of the protein by removing the metalloprotease domain [26]. Using this technique, they were able to induce the production of the ADAM10 mutant in selected tissues using tissue-specific promoters and at specific times during embryogenesis using the heat shock promoter. The mutant ADAM10 produced

phenotypes in the retina and the wing that were reminiscent of the Notch loss-of-function mutation. The "putative dominant negative" could be rescued by overexpression of the wild-type protein, arguing that the phenotype was specific to ADAM10 function. By expressing the DN in frog embryos, they also showed that the number of neurons was increased, suggesting that in vertebrates, the role of ADAM10 is conserved.

While our understanding of the function of ADAM10 has greatly improved since these early studies, the mechanism by which the proteolytically inactive ADAM10 protein may act as a dominant negative remains unclear. The simplest and most likely explanation is that the mutant binds to the substrate and competes with the active protein. Given the large overlap of substrates between the various ADAMs, it is likely that the dominant negative form of ADAM10 may also interfere with the function of related ADAMs expressed in the same cells and this should be kept in mind during the analysis of the phenotypes.

By specifically expressing the DN ADAM10 in neurons, Pan and colleagues were able to reproduce the axon extension phenotype obtained with the zygotic null mutant, further arguing that the mutant acted as a DN. Using the *Drosophila* S2 cells, as well as embryos expressing the DN and embryos with the null mutation, they showed that ADAM10 cleaved the extracellular domain of Notch and that in embryos lacking ADAM10 function the Notch receptor was not cleaved. The cleavage of Notch is essential for its activation and, therefore, loss of ADAM10 results in inactive Notch signaling. Interestingly, another study showed that in S2 cells the processing of Notch was not compromised by the ADAM10 DN, but that ADAM10 could process Delta and release a soluble fragment that retained the ability to bind to the Notch receptor, suggesting that this could promote signaling in the absence of cell contact [35]. They showed both that the DN ADAM10 and the loss of ADAM10 resulted in the absence of cleaved Delta protein in embryos. Cleavage of both Notch and Delta could explain how ADAM10 is required in the signal-receiving cells (Notch) and the signal-generating cells (Delta) for proper neurogenesis [32]. In fact, another study showed that a Kuzbanian-like protein (also related to ADAM10) was expressed in *Drosophila* embryos and appeared to process Delta in the wing imaginal disc [36]. In this study, they showed that cells that receive the Notch signal maintain a high level of Notch on their surface while reducing the level of Delta. In the absence of Kuzbanian-like, the level of Delta increases interfering with proper Notch signaling.

Thus, in *Drosophila* the receiving cell expresses both Kuzbanian and Kuzbanian-like, two proteins closely related to ADAM10. Kuzbanian processes Notch to activate the receptor while Kuzbanian-like cleaves Delta to prevent its signaling to neighboring cells. While this has not been demonstrated, it is likely that DN Kuzbanian may interfere with both functions, while the loss of one of the genes may or may not be compensated by the other. Until a clear understanding of the control of ADAM cleavage is achieved, we can only speculate about the function of individual ADAMs in developmental models. Evidence for the importance of ADAM10 in the cleavage of Notch and Delta in *Drosophila* have been confirmed by many studies in flies, nematodes and mice, making it the best-characterized signaling pathway involving an ADAM [26,35-48]. In *C. elegans*, several studies have confirmed the role of ADAM10 in Notch signaling and have also shown that ADAM17 can compensate, at least partially, for the loss of ADAM10 in cells that expresses both [39,45]. Surprisingly, while both ADAM10/Sup17 and ADAM17/ADM-4 genetically interact with Notch/Lin12 signaling, the absence of one or both of these genes does not produce the Notch phenotype, suggesting that, in the nematode, other proteases may also cleave Notch, or that Notch may also signal in a cleavage-independent manner.

Given the pleiotropic nature of Notch signaling during development, loss of ADAM10 function is likely to affect most developmental processes. While this was a strength for the initial

discovery of ADAM10 function, it also makes it very difficult to identify other functional substrates in the absence of proper Notch signaling.

3.2 Role of ADAM10 in organ morphogenesis

In *Drosophila*, ADAM10 is also required for heart morphogenesis. Loss of zygotic ADAM10 function results in an increase in cardioblasts and a decrease in pericardial cells [46]. This phenotype can be phenocopied by expressing the DN in dorsal mesoderm cells prior to the specification of cardioblast precursors, but not if the DN is expressed in the cardioblast, suggesting a role in the early cell specification. At later stages, cardioblast migration was not affected by ADAM10 loss-of-function but the morphology of the cells in the heart was.

3.3 ADAM10: a master controller of epithelium to mesenchyme transition (EMT)

The mouse model has provided answers and functional evidence about other embryologically-relevant substrates of ADAM10. Mice lacking ADAM10 die at embryonic day 9.5 with severe defects in the central nervous system (CNS) and both the dorsal (somitic) and ventral (cardiovascular) mesoderm [41]. Using *in situ* hybridization with Hes-5, a gene induced by Notch signaling, they further showed that Notch signaling was decreased in ADAM10 KO embryos. In addition, Delta-1 protein expression was found to be increased in these embryos, suggesting that ADAM10 in mouse embryos functions similarly to Kuzbanian to cleave both Notch (to activate) and Delta (to reduce).

In addition to the Notch phenotype, one of the most striking features of these embryos was their small size. In the epithelium, E-cadherin and N-cadherin proteins accumulated to abnormally high levels [49,50]. These proteins were found to be cleaved by ADAM10 to promote epithelium to mesenchyme transition (EMT) essential for both embryonic development and wound healing. In addition, the accumulation of Cadherins at the cell membrane sequestered β-catenin, decreasing its translocation to the nucleus and subsequent signaling. Using cell lines from the KO, ADAM10 was also shown to be a major contributor of EGF and β-cellulin shedding [51]. Defects in the processing of these growth factors are likely to contribute to the overall phenotype of the ADAM10 KO, but direct proof of this has not been shown. Other cell adhesion molecules, such as the protocadherin, PCDHγ, are cleaved by ADAM10 and could contribute to the neural phenotype by perturbing the "adhesive code" used during neuronal wiring [52]. While this has not been demonstrated, it is likely that ADAM10 cleavage of cell adhesion molecules, like Cadherins and protocadherins, also occurs in *Drosophila* and may be responsible for some of the phenotypes observed in the Kuzbanian-mutant embryos. In particular, phenotypes involving cell migration and neurite outgrowth could be due to defects in cell adhesion molecules or ephrin signaling (see **3.4**).

In the chicken, ADAM10 is widely-expressed and most abundant in epithelial structures. Knock down of ADAM10 with an antisense Morpholino oligonucleotide induces a thickening of the epithelium together with increased expression of N-Cam protein [53]. At the moment it is unclear if this phenotype is due to defects in the processing of cell adhesion molecules (N- and E-cadherin) or Notch or Delta. In addition, in quail, the metalloprotease inhibitor, GI254023X, which blocks ADAM10, was shown to prevent N-cadherin cleavage during neural crest emigration. This resulted in a reduction of β-catenin signaling and the inhibition of neural crest delamination from the neural tube [54]. These results provide additional evidence that ADAM10 cleavage of Cadherins may play an essential role in the developmental EMT in vertebrates. It would be interesting to see if this is also the case in invertebrates.

3.4 ADAM10 switches ephrins off in neurons

One more family of substrates for ADAM10 discovered in the mouse model is the ephrin. Two ephrin ligands, ephrin-A2 and -A5, have been shown to be cleaved by ADAM10. ADAM10

and ephrin-A2 are co-expressed on axons. When axons contact a cell expressing the Eph-A3 receptor, ephrin-A2 is cleaved by ADAM10, inducing a signaling cascade that promotes axon retraction. In the absence of ADAM10 the repulsion signaling cascade still occurs, but the axon remains attached to the repulsing cells as the ephrin/Eph interaction is not released [55]. In the case of ephrin-A5, ADAM10 is constitutively associated with the Eph-A3 receptor. When clustered ephrin-A5 is engaged with the Eph-A3 receptor, ADAM10 associates in trans via its cysteine-rich domain and cleaves the ephrin-A5 ligand, once again resulting in the repulsion and release of the axon [23]. While this has not been demonstrated in embryos, it is tempting to speculate that the loss of cleavage of ephrins by ADAM10 is, at least in part, responsible for axon extension defects both in mice and *Drosophila* lacking the ADAM10 protein.

3.5 ADAM17 is the master controller of EGF signaling

While ADAM17 is one of the most-studied ADAMs in mammals, there is only one report addressing the function of this protein in non-mammalian species. In *C.elegans*, ADAM17/ADM-4 null mutants are viable. They show a low-penetrance phenotype related to abnormal germ cell division. This phenotype is exacerbated in embryos lacking both ADAM10 and ADAM17, further demonstrating the functional redundancy of these two proteins [45]. At this point, it is unclear which of the ADAM17 substrates are involved in *C.elegans* development.

Mice lacking ADAM17 die perinataly and suffer from multiple defects in most organs [56]. The defects appear to occur from problems arising in the epithelial layer of each organ. Phenotypes include abnormal development of the skin, the eye, digestive tract, lungs and heart. Cells isolated from the KO mice have significantly less shedding of TNF-α, TGF-α, and the Erb-B4 receptor [56,57]. Mice lacking ADAM17 have enlarged hearts due both to increased cardiomyocyte proliferation and a lack of proper morphogenesis. Isolated hearts have a significant reduction in EGF signaling. It has been proposed that the function of ADAM17 in the heart is to regulate cell proliferation and cell organization during morphogenesis [57]. In the lung, lack of ADAM17 results in reduced organ size due to decreased cell proliferation of epithelial cells and a decrease in branching morphogenesis [58-60]. Interestingly, the ADAM17 protein is found in the plasma membrane of epithelial cells at their contact with the mesenchymal layer. Organ culture confirmed that branching morphogenesis was severely reduced in KO lungs and this could be rescued by adding exogenous TNF-α or EGF, suggesting that ADAM17 function during lung morphogenesis is as a sheddase mediating EGF signaling. This is also supported by the fact that the ADAM17 knock outs phenotypes are very similar to that of EGF receptor knock outs [59,60] as well as the triple knock outs for EGF, amphiregulin and TGF-α [61]. While in cell culture, ADAM17 can cleave many substrates (Figure 2), although the relative importance of each substrate during embryogenesis has not been established. For example, while ADAM17 can cleave Notch *in vitro* [48] and is essential for Notch processing in bone-marrow cells [62], none of the phenotypes observed in the mouse KO suggest that ADAM17 is essential for Notch function during early embryogenesis. Conditional knock out and rescue experiments expressing either the wild-type protein or the "shed" ligands in a tissue-specific manner should help further elucidate the relative contribution of ADAM17 substrates during embryogenesis.

4. The mesenchymal ADAMs, (Meltrins: ADAM9, 12, 13/33, 19)

The meltrins were originally cloned in an effort to identify proteins involved in cell fusion. ADAM12 (meltrin-α) and ADAM19 (meltrin-β) were found to be expressed in muscle precursor cells. In addition, ADAM12 was also expressed prior to myoblast fusion in the form of three main polypeptides. Based on their sizes, these polypeptides likely represent the precursor (including pro-domain), the mature protease (lacking prodomain) and a shorter form most likely lacking both the pro- and metalloprotease domains. Reduction of ADAM12 in C2C12 cells (a muscle cell line), using antisense approaches reduced cell fusion *in vitro*. In

contrast, overexpression of the short form lacking both pro- and metalloprotease domains increased cell fusion. Surprisingly, overexpression of the full length ADAM12 decreased fusion suggesting that only the truncated form was competent to promote cell fusion and that an excess of the pro-form may compete with endogenous short ADAM12 binding to elements of the cellular machinery [63]. These key experiments promoted the notion that the disintegrin domain of ADAMs became accessible as ligands for cell adhesion molecules following the removal of the metalloprotease domain similar to what was already known for the fertilins (ADAM1 and 2) at the surface of spermatozoids [5,64,65]. However, ADAM12 deletion in mice shows no obvious defect in myoblast fusion, demonstrating that ADAM12 is not absolutely essential for muscle development *in vivo*. While the ADAM12 knockout did not have the expected muscle phenotype, mice lacking ADAM12 showed approximately 30% perinatal mortality. About one third of the embryos lacking ADAM12 show some reduction of the interscapular brown adipose tissue, and in the neck and interscapular muscles. Cells isolated from the knock out mice showed reduced shedding of HB-EGF in response to phorbol ester stimulation [66]. While the ADAM12 loss-of-function experiments are relatively uninformative, gain-of-function experiments have produced some interesting results. In particular, study of ADAM12 proteins expression during myoblast development has revealed the presence of a secreted form of ADAM12 lacking the transmembrane and cytoplasmic domains. When human rabdocarcinoma cells were transfected with the secreted ADAM12 and introduced into nude mice, the tumors formed by the human cells were invaded by mouse myoblasts, suggesting that the secreted ADAM12 protein could recruit muscle precursor cells [67]. Similarly overexpression of ADAM12 in muscle improves muscle regeneration following injury and alleviates some of the symptoms of *mdx* mice (dystrophyn deficient) [68], suggesting a role for this protein in muscle regeneration. However, loss-of-function experiments show that mice lacking ADAM12 possess normal regeneration capacity [66]. Overexpression of ADAM12 in mice induces ectopic adipocytes resulting in increased weight [69]. Together with the knock out data, these data suggest that ADAM12 may participate in adipogenesis.

4.1 Are meltrins useless or replaceable?

The lack of an obvious phenotype in the ADAM12 knock out is one of the most frustrating characteristics of the meltrin subfamily of ADAMs. For example, there are two meltrins in *Drosophila*, D-meltrin and Mmd (Mind meld), but to date there are no phenotypes reported for either genes. In *C-elegans*, ADM-2 is the closest relative of the meltrins, and as with *Drosophila* there are no phenotypes associated with the loss of ADM-2 function during embryogenesis. In mice, loss of ADAM9, which is ubiquitously expressed, has no phenotype [70]. Furthermore, mice lacking ADAM9, 12 and 15 are viable and fertile [71]. Taken together these results suggest that either these genes have no function during embryogenesis or that other metalloproteases (ADAMs or others) can substitute for the absence of these proteins. It is hard to believe that genes expressed at significant levels in various tissues during embryogenesis do not have a function and, therefore, the second hypothesis seems more likely.

In the frog, three meltrins are expressed during early embryogenesis. ADAM9 is present maternally and in all cells [72], whereas ADAM13 and 19 are both expressed zygotically in very similar patterns, in the somitic mesoderm, the neural tube and cranial neural crest cells (CNC). In addition, ADAM19 is expressed in the notochord as early as gastrulation while ADAM13 appears to be absent from this structure ([73]; Neuner et al., submitted). Experiments using the proteolytically inactive form of ADAM13, as a DN, have shown that ADAM13 function is essential for the migration of the CNC [27]. Loss of the ADAM13 protein using a Morpholino AS approach has been reported to decrease CNC induction and migration in *Xenopus* [74], but we have not been able to confirm these results. In our hands, the ADAM13 MO efficiently eliminates the endogenous protein and interferes with gastrulation movements but has only marginal effect on CNC induction and migration (Cousin et al., in preparation,

McCusker et al., submitted). In contrast, elimination both of ADAM13 and 19 in the CNC completely inhibits CNC migration, while ADAM19 MO by itself has only marginal effects on migration but does decrease CNC induction (Neuner *et al.*, submitted, Cousin *et al.*, in preparation, McCusker *et al.*, submitted). These results confirm the potential compensation of meltrin ADAMs during early embryo development.

4.2 How does ADAM13 control CNC migration?

We have shown that ADAM13 can bind and cleave fibronectin *in vitro*, suggesting that it may interfere with cell-ECM interactions [27,75]. In addition, ADAM13 and ADAM9 cleave Cadherin-11, a cell adhesion molecule expressed in *Xenopus* CNC and known in mammals as a mesenchymal Cadherin. Loss of ADAM9 or ADAM13 results in an accumulation of Cadherin-11 in the embryo (McCusker *et al.*, submitted). Overexpression of Cadherin-11 prevents CNC migration [76] and this can be rescued by the overexpression of ADAM13. Similarly, expression of the cleaved, extracellular domain of Cadherin-11 can rescue migration in embryos overexpressing Cadherin-11, as well as those lacking ADAM9, 13 and 19 (McCusker *et al.*, submitted). This suggests that one of ADAM13 main functions in CNC is to cleave Cadherin-11, in order to release its extracellular domain and promote cell migration. This is reminiscent of ADAM10 cleavage of another adhesion molecule, L1. The extracellular domain of L1 binds to the $\alpha v \beta 3$ integrin and promotes cell migration [77,78]. Additional work will be needed to understand how the Cadherin-11 extracellular domain promotes cell migration. Another interesting aspect of Cadherin-11 cleavage by ADAM13 is that it occurs some distance from the plasma membrane, between the EC3 and EC4 domains, while cleavage of N- and E-cadherin by ADAM10 is near the membrane. This is significant because cleavage of N- and E-cadherin by ADAM10 is immediately followed by a second cleavage by a gamma-secretase, releasing its cytoplasmic domain and β-catenin, which immediately translocates to the nucleus and activates downstream genes including c-myc and cyclin-D1 [49,50]. In contrast, cleavage of Cadherin-11 by ADAM13 does not induce β-catenin signaling, suggesting that it can control cell adhesion and migration without modifying the signaling capacity of Cadherin-11 (McCusker et al., submitted). In *Xenopus tropicalis*, ADAM13 may also function in cleaving ephrin-B ligands to interrupt signaling. Negative control of ephrin-B signaling appears to be essential for head patterning and neural crest cell induction (DeSimone, personal communication). In mice, the knock out of ADAM33, the most likely homologue of ADAM13, shows no phenotype. There are no observable defects and the adults are fertile [79]. As with ADAM13, the extracellular domain of ADAM33 can be shed from the cell surface [75], and for ADAM33 the soluble form can induce angiogenesis *in vitro* and *ex vivo* (organ culture) [80]. Given our results with ADAM13, it would be interesting to produce multiple ADAM knock outs, including ADAM9, 12, 19 and 33, to investigate their role in development and migration of neural crest cells as well as the specification and fusion of myoblasts.

4.3. The exception, ADAM19

The only member of the ADAM family that shows a clear phenotype following knock out in mice is ADAM19. Mice lacking ADAM19 die soon after birth. Careful examination of ADAM19 null embryos shows severe defects in heart morphogenesis, including a ventricular septal defects and abnormal valve formation [81,82]. In addition, Kurohara and colleagues found defects in muscle due to decreased thickness of the myofibers, and some abnormal positioning of the thick preganglionic neuron bundle in the adrenal medula. Zhou and colleagues also showed that many of the capillaries were abnormal and eventually ruptured. Using β–galactosidase as a cell lineage tracer they showed that the cardiac neural crest of ADAM19 null embryos still migrated to the proper heart field. These observations were followed by another elegant study using the full "tool kit" of mammalian genetics [28]. In this report they studied, in detail, the contribution of cardiac neural crest cells and endothelial cells to heart formation in wild-type and knock out mice. By reintroducing the ADAM19 protein

specifically in cardiac neural crest or endothelial cells, they showed that ADAM19 was required in the cardiac neural crest to promote normal heart morphogenesis. They further showed that cardiac neural crest cells lacking ADAM19 were capable of migrating into the proper heart region but were unable to participate in the formation of the ventricular septum. While ADAM19 can cleave many substrates, including EGF ligands, the physiological substrate responsible for the observed phenotype is not known. As another testimony to redundancy, ADAM9-ADAM19 double knock outs have a more severe heart phenotype, including defects in the mitral valve [83]. Interestingly, in frogs, ADAM19 knock down also induces phenotypes. In particular, expression of transcript for the general mesoderm marker Brachyury is decreased in the notochord, while other dorsal mesoderm markers (Goosecoid and Chordin) are increased. At later stages of development, some tissue-specific markers are also decreased, including markers of neurons (N-tubulin), neural crest (Slug, Twist and ADAM11) and muscle (Myosin light chain). Together with a reduction in these markers, the organization of muscle precursors in the somites is perturbed, while the formation of the somite itself is not (Neuner et al., submitted). This suggests that, as shown in the mouse, frog ADAM19 possesses unique functions that are not compensated for by other related ADAMs. The analysis of multiple meltrin knock downs in frogs should help elucidate the overall redundancy and physiological substrates involved in early embryogenesis (Figure 3).

5. Non proteolytic ADAMs

These ADAMs have all the characteristics of the ADAM family including the metalloprotease domain, but the consensus active site sequence is missing, suggesting that they are not proteolytically active. Because of this particularity, the use of prototypical ADAM DN constructs was unavailable, and progress regarding their function has been slow. Fortunately, genetics has brought some invaluable information both in *C.elegans* and in mouse. The original work done on fertilin function during fertilization suggested that these non-proteolytic ADAMs functioned as ligands for the integrin family of receptors. While it is clear that most isolated disintegrin domains tested so far can promote cell adhesion mediated *via* selected integrins, the physiological role of these interactions remain controversial. Overexpression studies using full-length ADAM proteins have shown that they can regulate the activity of integrins, expressed in the same cells, with some selectivity, but the direct proof of this functional interaction in physiological conditions is lacking.

5.1 The nematode Unc-71/ADM1 controls cell migration

In *C.elegans*, Unc-71/ADM1/ADAM14 was identified during a search for potential fusion proteins (as proposed for ADAM function in myoblast and sperm/egg fusion). The protein was found in syncytial organs, sperm and sheath cells of sensory organs [84]. Mutation in the Unc-71 gene induces defects in axon guidance and axon fasciculation [85,86], as well as in the migration of hermaphrodite sex myoblast [87]. Detailed analyses of multiple alleles of Unc-71, show that most of the mutations are in sequences coding for the disintegrin and cysteine-rich domains of the protein. Subtle mutations in the disintegrin loop (D504 to Q) drastically affect protein function, suggesting that this region of the protein is important either for folding or for interaction with other proteins. Surprisingly, while the metalloprotease domain is likely inactive, rescue experiments demonstrate that this domain is essential for the proper function of the protein. Further experiments are needed to test whether this domain is required for the proper folding and export of the protein or for its function. Other rescue experiments showed that Unc-71 protein acts in a non cell-autonomously fashion in sexual myoblast migration, meaning that the protein does not rescue migration if it is expressed directly in the sexual myoblasts, but rather, needs to be expressed in cells along the pathway [85]. In addition, the presence of the transmembrane and cytoplasmic domains of the protein is required for the

rescue, suggesting that it helps guide cell migration by providing a permissive cue on the cell surface. This is also true for the function of Unc-71 in type-D motor axon guidance [85].

5.2 Non-proteolytic ADAMs in vertebrates: The "smart" ADAMs

In the mouse, non-proteolytically active ADAMs are expressed both in the central nervous system (CNS) and in the testis. We will not discuss the function related to fertilization as these have been described in detail elsewhere [11,88-91]. ADAM2, ADAM11, ADAM22 and ADAM23 are expressed in the CNS and have been knocked out with significant consequences.

Loss of ADAM2 in the CNS perturbs neuroblast migration along the rostral migratory stream to the olfactory bulb resulting in a smaller olfactory bulb. Both *in vitro* and *ex vivo* (in organ culture) experiments have shown that the migration of the neuroblasts was decreased. This was associated with an apparent decrease in cell/cell interaction. The defects in cell migration could be reproduced by addition of a peptide corresponding to the disintegrin loop of ADAM2 in the organ culture experiments, suggesting that the disintegrin domain is essential to promote or guide cell migration [92].

ADAM11 is expressed in the CNS of mice and both the CNS and cranial neural crest cells of frogs [72,93]. Mice lacking ADAM11 are viable and fertile with no major histological defects but show behavior defects related to learning, motor coordination and response to pain [94, 95]. The cellular basis for these defects is unknown.

Loss of ADAM22 and ADAM23 results in death shortly after birth (1 to 3 weeks) due to tremors and ataxia [96,97]. For ADAM22, detailed analysis of the brain of the homozygotes revealed a normal organization with the exception of a deficit in myelination of the peripheral sciatic and trigeminal nerves while the myelination of other nerves in the spinal chord and the brain was not affected. Myelination of peripheral nerves depends on the function of Schwann cells. These cells were more abundant in the mutant embryos suggesting that they did not stop dividing and failed to differentiate to produce myelin. In support of this hypothesis, ADAM22 gene is not expressed in highly proliferative gliomas (cancer), and artificial expression of the protein in these cells can inhibit proliferation. Inhibition of cell proliferation could also be achieved by incubating cells with a bacterially expressed ADAM22 disintegrin domain. In addition, overexpression of integrin linked kinase (ILK) in glioma cells could prevent the inhibition of proliferation suggesting that the disintegrin domain was reducing cell proliferation by binding to an integrin at the cell surface [98]. Finally, ADAM22 was also found to be present in the post-synaptic membrane where it could act as a receptor for LGI1, a secreted protein involved in epilepsy. A mutated form of the LGI1 protein, found in autosomal dominant partial epilepsy with auditory feature, failed to bind to ADAM22 [99]. It is tempting to speculate that the phenotypes associated with the loss of ADAM22 in mice may be related to its role as a receptor for LGI1.

In summary, non-proteolytic ADAMs are involved in nervous system formation both in vertebrates and invertebrates. Their functions are likely to involve the regulation of cell adhesion to promote axon guidance, neuroblast migration and Schwann cell differentiation. The identification of ligands for non-proteolytic ADAMs will be essential to understanding the mechanisms by which these ADAMs perform their function.

6. Conclusions

ADAMs are essential proteins involved at every step of embryo development. They control cell proliferation, cell specification, cell migration, axon elongation and organ morphogenesis (Figure 4). They are so essential that built-in redundancy and functional overlap is the rule,

with only a few exceptions. Attributing "the" functional substrate for each ADAM in each cellular context will be the next great challenge.

Acknowledgments

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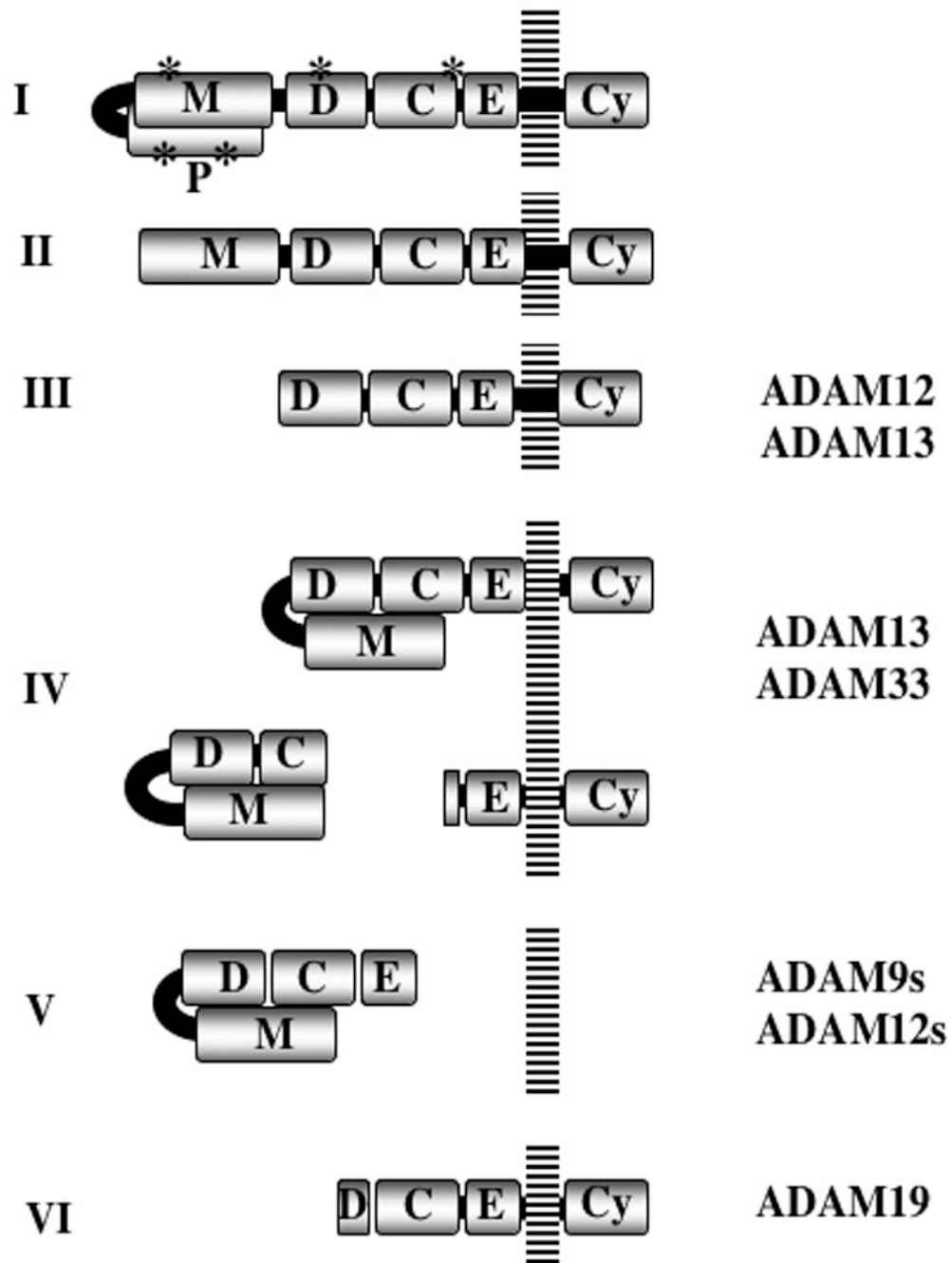
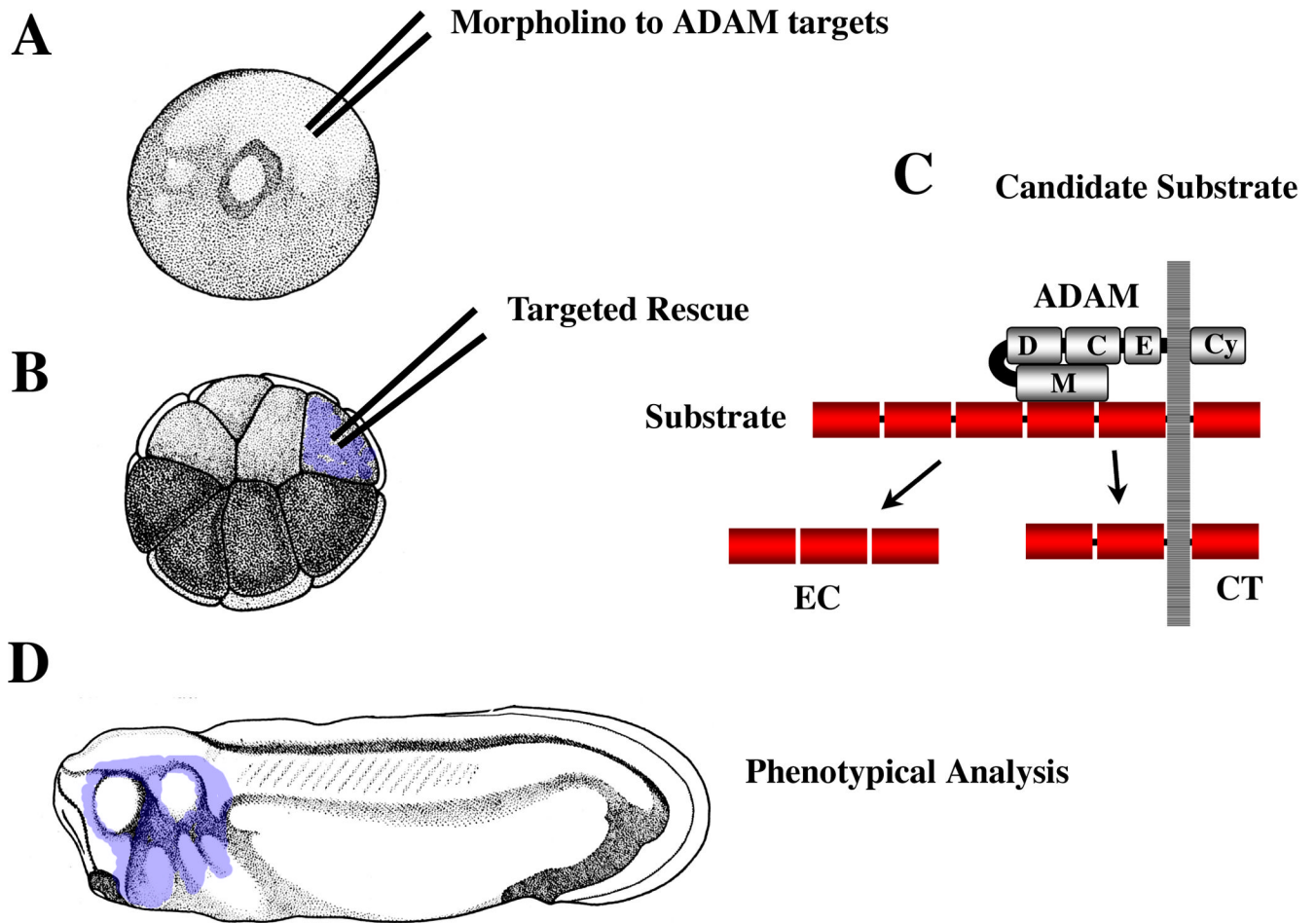**Figure 1. Schematic diagram of ADAM protein organization and processing**
(I) ADAMs are single-pass transmembrane proteins composed of a pro-domain (P) a
metalloprotease domain (M), a disintegrin domain (D) a cysteine-rich domain (C) an EGF
repeat (E) and a cytoplasmic domain (Cy). (II) The pro-domain of ADAMs is removed by furin
cleavage either during the transit through the trans golgie network or at the cell surface
producing the metalloprotease active form. (III) In some cases, a second cleavage occurs
removing the metalloprotease domain and potentially "unmasking" the disintegrin domain to
promote integrin binding [63,73]. (IV) Additional shedding has been reported for both
ADAM13 and ADAM33 whereby the metalloprotease domain is shed with the disintegrin and
cysteine-rich domains [75,80]. (V) A similar soluble active metalloprotease can be obtained

by alternative splicing as shown for ADAM9s and ADAM12s [67,100]. (VI) Alternative splicing can also generate a proteolytically inactive ADAM, anchored in the membrane as shown for ADAM19 [101].
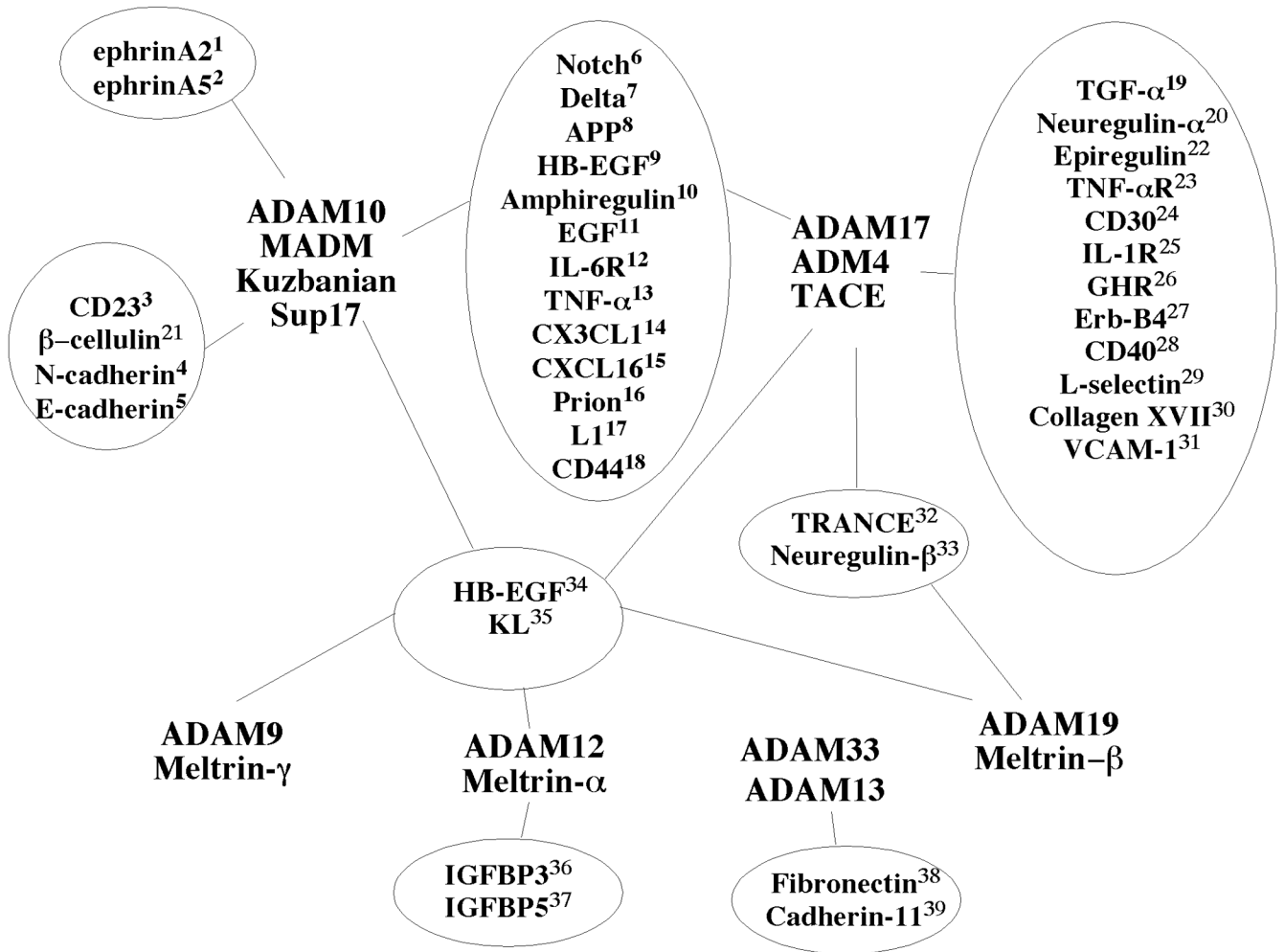
Figure 2. ADAM proteolytic substrates
Diagram of selected ADAMs and substrates. The potential overlap between different ADAMs is presented. The number of potential substrates and the overlap makes the analysis of phenotype very difficult. 1 [55], 2 [23], 3 [102], 4 [49], 5 [50], 6 [26], 7 [35], 8 [103], 9 [104], 10 [105], 11 [71], 12 [106], 13 [107], 14 [108,109], 15 [110,111], 16 [112], 17 [78], 18 [113,114], 19 [115], 20 [116], 21 [51], 22 [71], 23 [115], 24 [117], 25 [118], 26 [119], 27 [120], 28 [121], 29 [115,122], 30 [123], 31 [124], 32 [125-127], 33 [83,128,129], 34 [83, 130-132], 35 [127,133], 36 [134,135], 37 [134], 38 [27], 39 McCusker et al., submitted.

A Morpholino to ADAM targets

C Candidate Substrate

ADAM

Targeted Rescue

B

Substrate

EC

CT

D

Phenotypical Analysis

Figure 3. How to study ADAM function during development
Example of the strategy employed in *Xenopus* to test the function and potential substrate of
ADAM proteases during development. The ADAM protein translation is inhibited by injection
of a Morpolino oligonucleotide into the fertilized egg (A). At the blastula stage, mRNA
encoding the wild type protein or various mutant lacking selected domains can be injected in
a cell that will give rise to a specific tissue, for example the cranial neural crest cells (B). A
lineage tracer is co-injected to compare the behavior of these cells to the rest of the embryo
missing the ADAM protein. If an appropriate substrate is identified (C), the putative cleavage
product (EC) can be produced and injected to test its ability to rescue the loss of ADAM
phenotype. Phenotypical analysis can be performed at the tailbud stage (D) using the lineage
tracer and tissue specific marker to determine the position and differentiation of the injected
cells progeny. Similar techniques can be used in genetic models using tissue specific promoter
rather than targeted injection.
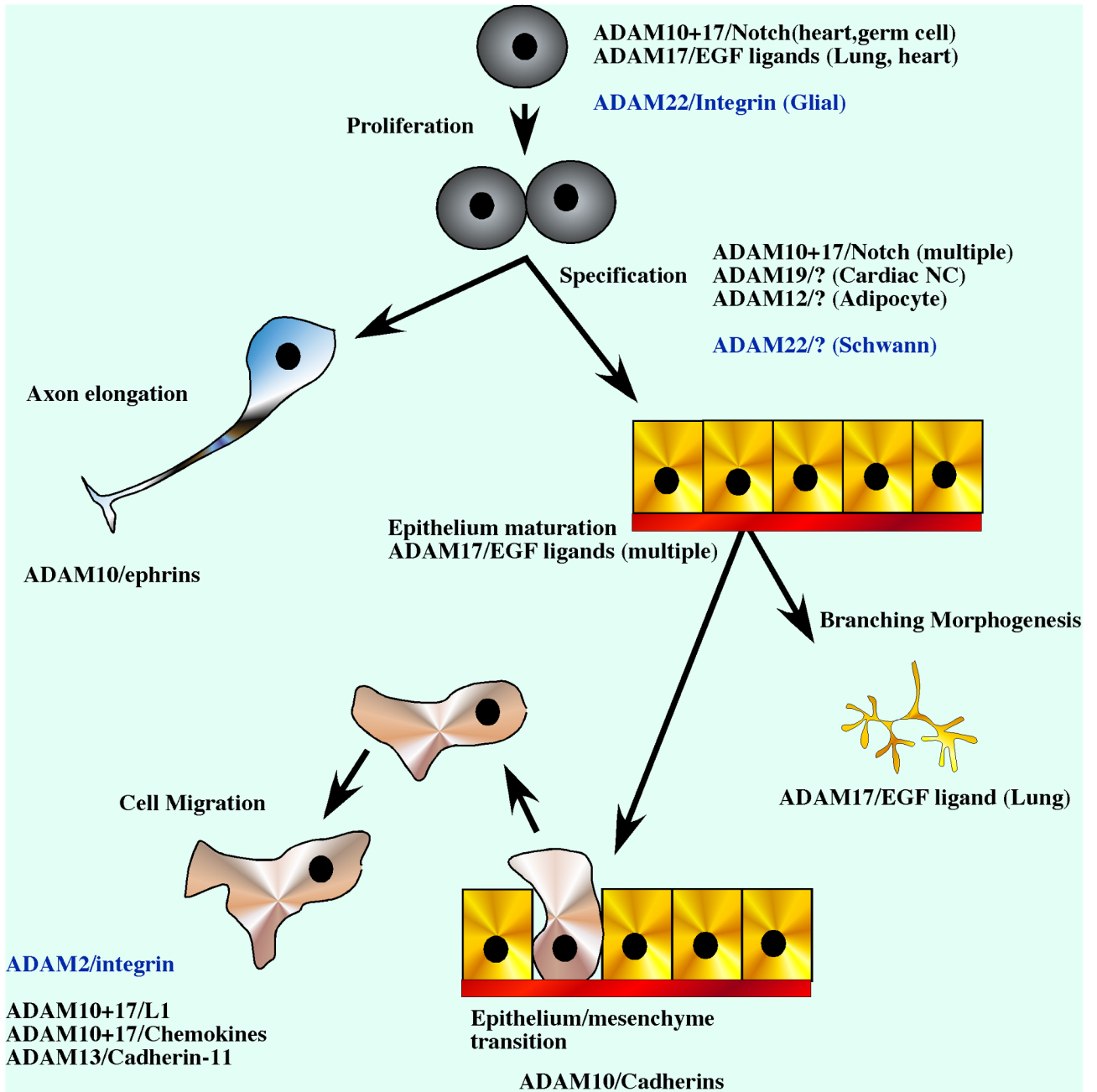
Figure 4. ADAM function in Development
ADAM proteins are involved at every step of embryogenesis. They can control cell proliferation, cell specification and differentiation as well as various aspects of morphogenesis. ADAMs that have been implicated with each step are indicated together with the substrate if it is known. In parenthesis the cell type or tissue in which the function was shown is indicated. Non-proteolytic ADAMs are in blue.