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### Mathematical modeling of metal ion homeostasis and signaling systems

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## Chapter 1 General Introduction

The desire to understand life is as old as mankind [229]. Although the incredible diverse nature of living systems, often called biocomplexity, still forms a formidable challenge for computational science, it is believed that the method of computational and information science will help to establish the next generation biology [30,69]. In this post-genome era, the modeling and simulation of cellular processes such as signaling and metabolic pathways become increasingly important. The overwhelmingly great complexity and sophistication of these processes exceed our intuition and tax the most advanced mathematical and computational means [229]. The ability of mathematical and computational models to integrate collections of observations and connect them to a higher level of biological organization facilitates the understanding of cell function and physiological phenomena [30,37,166,229].

Metal ions such as calcium and zinc play critical roles in biological cells. For example, in eukaryotic cells,  $\text{Ca}^{2+}$  functions as a highly versatile intracellular messenger regulating a myriad of cellular processes such as proliferation, muscle contraction and neurotransmitter release [20-22,174,231]. Zinc serves as a structural component or catalytic cofactor in a large number of proteins such as RNA polymerase and zinc finger proteins and it also has very important signaling role [26,42,66,77,172,190,219]. The intracellular concentration of metal ions is usually under tight control through corresponding homeostasis systems involving coordination between ion uptake, distribution, storage and efflux [123,182]. Mathematical modeling and computational simulations are required for the understanding of metal ion homeostasis and signaling networks which is of fundamental importance in systems biology and has many applications in medicine (e.g., the understanding of zinc signaling in tumor cells will help devise therapeutic strategies to attenuate tumor growth, see Section 1.3.4.7. The understanding of calcium signaling in cardiac myocytes is critical for devising therapeutic drugs to treat diseases such as cardiac hypertrophy and heart failure, see Section 4.1). In this thesis, we will report some progress which we have made in this important field.

### 1.1 System Biology and Its Grand Challenges

*"Every object that biology studies is a system of systems." Francois Jacob (1974).*

Systems biology, as its name indicates, is an emergent field focusing on systematic study of complex interactions in biological systems [35,107,222,251]. According to Lilia Alberghina (University of Milano-Bicocca, Italy): "There is a growing awareness in medical science that biological entities are 'systems' -- collections of interacting parts" [252]. For example, in metal ion homeostasis and signaling systems, various proteins such as ion transporters, sensors and transcription regulators interact with each other and work together to achieve the tasks. However, it is only very recently that the systems approach becomes in fashion because the birth of functional genomics, the development of novel numerical methods, and the abundance of computational resources (due to the

usage of facilities for supercomputing, grid computing and cloud computing, etc.) make it possible to achieve system-level understanding of biological processes [35,107,222].

### 1.1.1 A Bit History of Systems Biology

The perspective of integration used by systems biology can be traced back to the Greek philosopher Aristotle (384–322 B.C.) who stated: "the whole is something over and above its parts and not just the sum of them all" [220]. As a newly emerged biological research field, systems biology finds its roots in the quantitative modeling of enzyme kinetics, the numerical methods developed to study neurophysiology, control theory and cybernetics [251].

The Austria-born biologist Ludwig von Bertalanffy (1901-1972) can be seen as a precursor of systems biology because of his "general systems theory". The famous Hodgkin-Huxley model (1952) of the squid giant axon [105], developed by British neurophysiologists Alan Lloyd Hodgkin (1914-1998) and Andrew Fielding Huxley (1917- ), described a cellular function (membrane action potential) emerging from the interaction between two components (a potassium channel and a sodium channel) and can therefore be seen as the beginning of (computational) systems biology [251].

System theorist Mihajlo Mesarovic (1928- ) launched the formal study of systems biology as a distinct discipline in 1966 with an international symposium entitled "Systems Theory and Biology." Systems biology emerged as a movement in its own right around year 2000, symbolized by the establishment of Institutes of Systems Biology in Seattle and Tokyo [251]. Since then, various ambitious research initiatives and projects under the flag of systems biology have been launched such as the Silicon Cell Initiative (Amsterdam, the Netherlands) [248], the Virtual Physiological Human (VPH) Initiative (Multi-national) [257] and the IUPS Physiome project (Multi-national) [247].

### 1.1.2 Biological Complexity

Biological systems are often claimed to be dauntingly complex. Biological complexity is closely related to the characteristically hierarchical structure of biological systems [199,253]. The recognizable structure levels (from top to bottom) include ecosystem, community, population, organism, organ system, organ, tissue, cell and molecule. In this hierarchical organization, each higher entity is composed of numerous lower entities with intricate linkages of interactions to keep them together<sup>1</sup>. For example, a single *E. coli* cell consists of thousands of molecules including 6000-10,000 proteins, 2000 metabolites, 5000 mRNAs and 4000 genes with countless interactions among them to construct extremely complex networks [213].

In addition to the intricate interactions of biological entities on the same level that constitute the main source of biological complexity, another source of biological complexity originates from the constant interplay between events at different levels [253].

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<sup>1</sup> With the exception that single celled organisms don't contain tissue and organ level entities.

Such interplay may happen between events at extremely different time and space scales. For example, the course of evolution can be changed due to a unique molecular event such as a single mutation. On the other hand, the change of global environment (e.g., global warming) can be very influential in determining the biochemical transformations of ordinary cellular metabolism (e.g., those transformations which lead to coral bleaching) [201].

### 1.1.3 Computational Challenge and Other Challenges

Biological complexity imposes grand challenges to researchers in the field of systems biology, the first of which is experimental. To accurately simulate biological processes, one of the critical steps is to retrieve the correct data. New generations of high-resolution and high-throughput experimental equipments are needed to measure the properties of the molecules in biological cells, visualize their activity, etc. For example, in metal ion homeostasis and signaling systems, new technical devices are needed to determine various rate constants of the relevant protein-protein interactions. New imaging techniques need to be developed to monitor the concentration change of metal ions in biological cells. The lack of powerful visualization and image reconstruction methods has been one of the major impediments to studying the role of metal ions in various human diseases such as the role of  $Zn^{2+}$  in Alzheimer's disease [31,78,192,211,219].

The second grand challenge is computational [72,122]. For example, to achieve system-level understanding of biological cells, we need to model and simulate the dynamic behavior of gene regulation networks, intracellular signaling pathways, metabolic networks, etc [210,213,228]. To build an integrated human physiology, we must combine data from many related areas including *genome*, *proteome*, *metabolome*, and *physiome*, which embodies knowledge about genes, proteins, metabolic processes, and physiology. To handle such huge amount of data of great complexity, new modeling approaches and computational methods need to be developed and impressive computational resources are essential [72,118,199,222].

The third grand challenge is the collaboration. The research in the field of systems biology is highly interdisciplinary. To approximate the real biological systems using computational models and simulations, close collaborations between experimentalists, mathematicians, physicists, biochemists and computational scientists are necessary. Good communication is extremely important for successful collaboration. Very often, molecular biologists complain that their colleagues responsible for computational research do not understand the biological terms in their words, whereas computational scientists often complain that their computational methods are often misunderstood by their biologist colleagues.

In addition, there seems to be a paradox related to biological data: on the one hand, we have seemingly over-opulent data, many of them are now stored in all kinds of databases [246,249,250]; on the other hand, we frequently face the situation of data scarcity when doing modeling [204]. For example, to model the *in vivo* dynamics of zinc homeostasis system in *E. coli*, we will find that besides the lack of many kinetic rate constants, even

the most basic data such as the exact value of cytosolic zinc concentration is missing because of the limitation of the current technology [163], which further illustrates the experimental challenge of systems biology. In some other cases, the measurement of missing data can be achieved with the help of various experimental groups that have corresponding technical equipments, which further illustrates the challenge of collaboration. Thus the motivation of finding the missing data makes mathematical and computational modeling a good way of promoting the data collection process and systematically organizing the data.

#### **1.1.4 Prize for Efforts: The Golden Fleece**

The prizes to be attained after dealing with the above mentioned grand challenges are immense. The mostly direct prize will be in medical science. Systems biology has the potential to have general profound effects on the healthcare and medical science ranging from in silico drug design and testing to individualized medicine by using model-based experimentation techniques. Complex diseases such as diabetes, heart failure, cancer, and metabolic syndrome, for which there are currently no cures, can be predicted and treated as systems biology progresses [14,222,252]. As we will introduce later, metal ion homeostasis and signaling processes are closely related to many human diseases (for example, the accumulation of copper in body tissues can cause the symptoms of Wilson's disease [209,251]; the shortage of potassium in body fluids can lead to cardiac arrhythmia [251] and calcium signaling network in cardiac myocytes is responsible for controlling cardiac hypertrophy). Thus the understanding of these processes is certainly necessary and critical for treating these relevant diseases.

The second prize to be attained will be in agriculture and material science. According to Uwe Sauer (the Institute of Molecular Systems Biology, ETH Zurich, Switzerland): (a systems-perspective could) "open up entirely new options for the production of chemicals, food products and in plant breeding" [252]. For example, the thorough understanding and accurate simulation of biomineralization processes in the organisms such as corals and sponges through systems biology approach will potentially lead to the manually-controlled production of minerals through biological organisms. Since biomineralization processes are often closely related with metal ion homeostasis and signaling processes (for example, calcium calcification in coral is based on calcium homeostasis in cells in various coral tissues), mathematical modeling of the later processes will be certainly quite necessary for understanding the former ones [6].

The third prize to be attained will be inspiration of new theoretical sciences and new directions in relevant existent sciences and their applications in engineering. In the past centuries, investigations on biological problems had promoted the birth of many sciences and new computational methodologies [10,73,151,203,251]. For example, the mathematical modeling of natural, biological processes has resulted in the birth of an interdisciplinary field of study named as mathematical biology [65,151,178]. Very recently (actually in 2002), the systems biology study of complex biological networks has led to a new theory named as network motif theory (see Chapter 6) [10,11]. It is natural to anticipate that such trends will continue. In order to tackle the numerous open

questions (e.g., protein folding, the encoding of calcium signaling specificity, reaction-diffusion systems with boundaries of complex geometry, etc.) related to complex biological phenomena, new theoretical sciences (including new mathematics, new chemistry, etc.) and new computational methods are most likely required [222].

Finally let me end this section with an analogy with a well-known Greek mythology: Argonauts and Jason sought for the Golden Fleece. If we think of the grand challenges of systems biology as the great dangerous things met by the Argonauts and Jason on their voyage and adventures (the harpies, the clashing rocks and the sleepless dragon, etc.), what we need to do is to build a strong team of Argonauts composed of excellent biologists, mathematicians, physicists, biochemists and computational scientists, whose diligent, continuous and collaborative works will make them conquer all the difficulties on the way and eventually acquire the golden fleece.

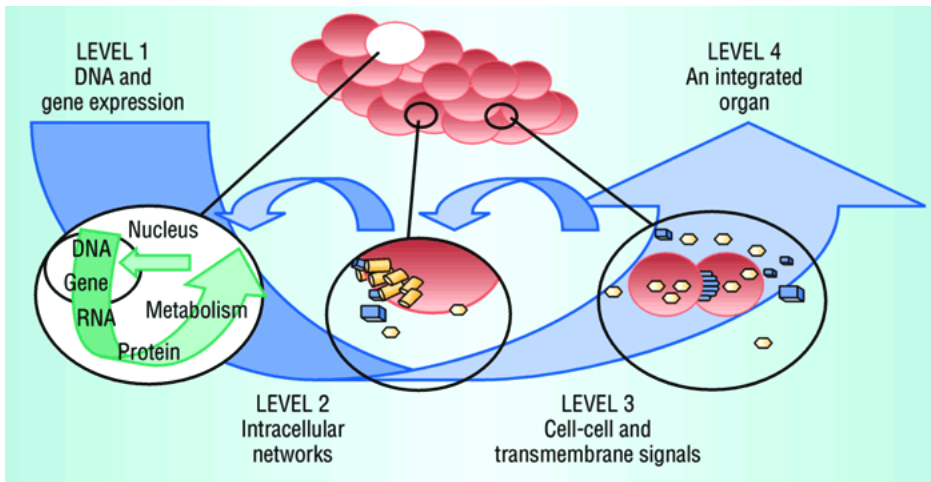
## **1.2 Mathematical Modeling**

Models are used as abstractions of reality whose representation includes diagrams, laws, graphs, equations, plots, etc [72,80]. A mathematical model is an abstract model that uses mathematical language to describe a system [251]. Although qualitative models such as boolean networks can be useful for describing systematic structures and processes, in this thesis we will mainly focus on quantitative mathematical models.

### **1.2.1 The Role, Levels and Strategies of Modeling**

Modeling lies at the heart of systems biology [72,80]. Mathematical models and their computer simulations can help us to understand the involved dynamics of complex interactions of biological structures (or entities), based on which the system-understanding of biological systems becomes possible. Modelers start from experimentally acquired data and observed phenomena, make assumptions and devise hopefully useful models, which can make predictions of the behavior of studied systems and these predictions are further validated by new experiments, the results of which are used to help improve the models. Such iterations between experiments and models are the only possible way leading to realistic models, which also illustrates the necessity of close collaboration between experimentalists and modelers [72].

The levels of models are closely related to the hierarchy of biological systems. As shown in Fig. 1.1, models at the most fundamental level are those for molecules such as DNA (genes) and proteins. Models at intermediate levels include those for describing intracellular networks, cell-cell and transmembrane signals. Models at the top levels consist of those for tissues, organs, organisms and ecosystem, etc.



**Figure 1.1. Different levels of models in systems biology.** From bottom to top are models for DNA and gene expression, intracellular networks, cell-cell and transmembrane signals and integrated tissue or organ. Even higher levels of models such as models for populations and ecosystem are not shown here (this figure is taken from Ref. 72).

As abstraction of certain aspects of complex biological systems, models always need judicious simplification which means a trade-off between simplicity and accuracy of the model. The strategies of modeling include simplification, construction and integration. Occam's Razor is a very useful principle for modeling, which means that among models with roughly equal predictive power, the simplest one is the most desirable [72].

## 1.2.2 Approaches of Mathematical Modeling

Mathematical models in biology mainly include difference equations, ordinary differential equations and partial differential equations [65,67,151,178]. Difference equations are special recurrence relations which define recursive sequences of numbers, which are widely used in population biology because the number of individuals is essentially discrete. For example, the Nicholson-Bailey model is a theoretical model for host-parasitoid systems using difference equations [65]. A more well-known example is that of the dynamic growth of a rabbit population which can be described by a concise difference equation ( $P_{n+1} = P_n + P_{n-1}$  for  $n > 0$  with  $P_0 = P_1 = 1$  where  $P_n$  denotes the number of rabbit pairs at the end of the  $n$ th month) leading to the famous Fibonacci sequence.

However, the majority of mathematical models in biology belong to differential equations which have been used for modeling both discrete and continuous biological processes. For example, the well-known Lotka-Volterra model consists of two ordinary differential equations for describing interactions of predator-prey populations. The Hodgkin-Huxley model consists of four ordinary differential equations for describing the dynamics of membrane action potential in a squid axon which is essentially continuous [105]. As we will see later, in many cases, the main gradients of systems biology such as feedback controls and enzyme kinetics can be expressed into the form of differential equations.

Ordinary differential equations (ODEs) describe the quantitative relations between variables and their derivatives, whose classical examples include the above mentioned two models (the Lotka-Volterra model and the Hodgkin-Huxley model). To a first approximation, many biological reaction networks can be described mathematically by a set of ODEs that track the effects of the simultaneously occurring reactions. The ODE method is extremely suitable and effective for modeling complicated intracellular networks (e.g., signaling networks and metabolic pathways) in relatively small cells where certain spatial effects (e.g., diffusion) can be neglected [69,188,213].

In other cases when spatial effects are important, partial differential equations become a more amenable tool for approximating biological systems. Examples of PDE models in biology include models for blood flow, cell motions, traveling calcium waves, chemotaxis and pattern formation [65,69,71,151]. Compared with ODE models, PDE models are usually much more difficult for numerical simulations. The irregular boundaries (e.g., those composed of the membranes of cells and/or cellular organelles) add much to this difficulty [112].

According to their properties, many mathematical models can be classified as linear vs. nonlinear, deterministic vs. probabilistic (stochastic), static vs. dynamic, lumped parameters (for homogeneous models) vs. distributed parameters (for heterogeneous models, typically represented by PDEs) [251]. In this thesis, we restrict ourselves to nonlinear ODE models, the properties of which will be further illustrated in Section 1.5.3.

### **1.2.3 Multi-Scale Modeling**

The hierarchical structure of biological systems determines the necessity of multi-scale modeling, especially when we want to achieve such ambitious goals such as predicting and treating human disease based on molecular-level knowledge [156,157,199,204]. As mentioned before, one major source of biological complexity originates from the constant interplay between events at different levels of biological systems with extremely different time and space scales [253]. Multi-scale modeling is necessary for capturing and reproducing the dynamic characteristics of such constant interplay by integrating information from lower levels (molecule and cell) to higher levels (tissue, organ, organ system and organism, etc.). Multi-scale modeling will be the ultimate modeling framework of systems biology and is expected to summarize knowledge (including physiology and molecular biology) of the broad biological context in a quantitative manner. The classical 19th century problems in physiology and disease will be addressed by the 21st century molecular technologies and by adopting the systems biology approach based on multi-scale modeling [204].

## **1.3 Metal Ion Homeostasis and Signaling Systems**

The relation between metals and living organisms is complicated. On one hand, many metals are essential for life as macronutrients or micronutrients and play important roles in its metabolism and growth. Micronutrients such as Co, Cu, Cr, Fe, Mn, Mo and Zn are



needed for human in small quantities (generally less than 100mg/day), which are also referred to as trace elements whose average concentrations are less than 100µg/g in the human body [123,251]. Macronutrients such as Na, Mg, K and Ca are needed in larger quantity and generally found in group IA and IIA of the periodic table. Insufficient intake of essential metals including macronutrients or micronutrients results in diseases or growth retardation [123]. On the other hand, metals are probably the oldest known toxins for life. Excessive intake of essential and some non-essential metals (e.g., Pb, Cd and Hg) results in toxicity. In this regard, highly regulated metabolic pathways (i.e., metal ion homeostasis systems) have been developed by living organisms to maintain essential or non-essential metals at optimal concentration ranges whereas detoxification mechanisms are used for many of the non-essential toxic metals [123,153].

### 1.3.1 Functions of Main Essential Metals

The major functions of main essential metals are listed in the following Table 1.1. An interesting observation may be that Al (aluminum), the most abundant metal in the Earth's crust is not in this list, the reason of which is that aluminum has no known function in living cells [251]. Another important fact worthy of notice is that Na, which is an essential macronutrient for human and other animals, is not needed by the plant [251]. This observation indicates the different requirements for particular metals in different kinds of organisms.

**Table 1.1: Major functions of eleven metals which are essential for human [236,251]**

Metal Symbol	Name and Ion Forms	Major functions in life
Ca	Calcium (Ca <sup>2+</sup> )	the most ubiquitous intracellular second messenger carrying signals to regulate a myriad of biological processes including proliferation, muscle contraction, neurotransmitter release and cell apoptosis; a major structural element in bones, calcified cartilage, teeth and shells
Mg	Magnesium (Mg <sup>2+</sup> )	help maintain normal muscle and nerve function; keep heart rhythm steady and bones strong; support a healthy immune system; help regulate blood sugar levels and promote normal blood pressure; needed for healthy teeth and energy metabolism; essential to the basic nucleic acid chemistry of life; a cofactor for ATP (adenosine triphosphate) and a number of enzymes; a regulator of ion channels; required for the structural integrity of numerous proteins and nucleic acids; a major component of chlorophylls in plants
Na	Sodium (Na <sup>+</sup> )	important for regulation of blood and body fluids; important for transmission of nerve impulses; important for muscle function and heart activity; having certain metabolic functions (please note that sodium ion is not needed by plants)
K	Potassium	work with sodium to maintain the body's water balance, thus important for regulation of blood and body fluids; important for

	(K <sup>+</sup> )	transmission of nerve impulses; important for muscle function and heart activity; help the kidneys function normally
Fe	Iron  (Fe <sup>2+</sup> , Fe <sup>3+</sup> )	necessary for formation of hemoglobin, brain development and function, regulation of body temperature, muscle activity and catecholamine metabolism; essential for a healthy immune system and hair growth; structural component of cytochrome proteins and some other proteins; contributing to redox reactions in the iron-sulfur clusters of many enzymes such as nitrogenase
Co	Cobalt  (Co <sup>2+</sup> , Co <sup>3+</sup> are most prevalent)	a central constituent of the vitamin B <sub>12</sub> , work with it to prevent anemia and ensure the health of the nervous system
Cr	Chromium  (Cr <sup>3+</sup> is essential and Cr <sup>6+</sup> is very toxic. All oxidation states from I to VI exist)	involved in amino acid transport and breakdown of glycogen and lipids; essential in order for insulin to function and important in the metabolism of fats and carbohydrates; stimulating fatty acid and cholesterol synthesis; an activator of several important enzymes
Cu	Copper  (Cu <sup>+</sup> , Cu <sup>2+</sup> )	important for synthesis of hemoglobin, proper iron metabolism, and maintenance of blood vessels; vital in making elastin, a chief component of the elastic muscle fibers found throughout the body; needed to build strong bones, connective tissue and joints; an essential components of macromolecules such as metalloenzymes and copper-based pigments; used for biological electron transport
Mn	Manganese  (Mn <sup>2+</sup> is most stable, although oxidation states from I to VII exist)	needed for bone development and maintenance of strong bones; helping to activate enzymes that are necessary for the body's proper use of biotin, B1 and vitamin C; important in the utilization of thiamine and the formation of thyroxine; cofactor of enzymes such as arginase and ligases ; component of the oxygen evolving complex (OEC), a water-oxidizing enzyme contained in chloroplast membrane in plants
Zn	Zinc  (Zn <sup>2+</sup> is the prevalent form, Zn <sup>+</sup> is rare)	structural component or catalytic cofactor in many proteins (e.g., zinc finger proteins; it is estimated that ~3000 proteins in human contain zinc); having signaling roles and functioning both as an intracellular second messenger and secreted signaling molecule in synaptic transmission, etc.; activator of certain enzymes such as carbonic anhydrase; playing role in olfaction

Mo	Molybdenum  (Mo <sup>6+</sup> , Mo <sup>5+</sup> , Mo <sup>4+</sup> , Mo <sup>3+</sup> , Mo <sup>2+</sup> , Mo <sup>+</sup> )	a cofactor for a number of enzymes that catalyze important chemical transformations in the global carbon, nitrogen, and sulfur cycles, three of which (sulfite oxidase, xanthine oxidase and aldehyde oxidase) are in human
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### 1.3.2 Metals-Related Human Diseases

As mentioned before, deficiency of essential metals in human can lead to lots of diseases. For example, a shortage of potassium in body fluids may cause a potentially fatal condition known as hypokalemia whose symptoms include muscular weakness, lack of energy, muscle cramps, stomach disturbances and occasional cardiac arrhythmias, etc. Long-term calcium deficiency can result in osteoporosis, a disease of bone leading to an increased risk of fractures. Zinc deficiency can cause hair loss, skin lesions, diarrhea, wasting of body tissues and malfunctions of eyesight, taste, smell and memory. The symptoms of chromium deficiency include severely impaired glucose tolerance, a loss of weight, and confusion. Iron deficiency is the most common form of nutritional deficiency and it can lead to a disease named as iron deficiency anemia which is characterized by pallor, fatigue and weakness [251].

On the other hand, excessive accumulation of essential metals in human body can result in a number of other diseases. For example, excessive intake of copper can result in the inhibition of certain important enzymes which leads to vomiting, diarrhea, hemolytic anemia, anuria and cirrhosis. Moreover, the symptoms (including mild cognitive deterioration and clumsiness and liver disease) of Wilson's disease, which is also referred to as inherited copper toxicity<sup>2</sup>, are caused by an accumulation of copper in human tissues and organs (especially in brain and liver). Miners can go mad with chronic exposure to manganese dust due to impaired motor skills and cognitive disorders caused by manganese poisoning [251].

Similarly, excessive intake of non-essential metals such as Pb (lead), Cd (cadmium) and Hg (mercury) can be toxic to human. Lead (Pb) poisoning works by the inhibition of some important enzymes which causes ineffective heme synthesis and subsequent microcytic anemia, a disease characterized by small red blood cells. Symptoms of mercury poisoning typically include sensory impairment (vision, hearing and speech), disturbed sensation and a lack of coordination. Inhalation of cadmium-containing fumes can result initially in metal fume fever but may progress to chemical pneumonitis and death [251]. Cadmium and several cadmium-containing compounds can also induce many types of cancer. It has been found that cadmium toxicity may result from the great similarity between cadmium and zinc so that cadmium (usually stronger than zinc in

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<sup>2</sup> Wilson's disease is an autosomal recessive genetic disorder due to mutations in the ATP7B gene encoding a copper ATPase (see Table 1.3). In Wilson's disease, copper accumulates in tissues and this manifests itself with neurological symptoms and liver disease [209,251].

binding ability) competes with zinc for the binding of zinc-binding proteins (in particular, proteins that contain zinc finger protein structures) [123].

### 1.3.3 Metal Ion Homeostasis Systems

Metal ion homeostasis systems are highly regulated metabolic pathways used by living organisms to maintain metals at optimal concentration ranges, the values of which may vary much for different metals in different sorts of organisms. Moreover, the metal ion concentration in the cytoplasm of biological cell can be quite different from that in the extracellular fluid and from those in intracellular membrane-bounded compartments. For example, the following table shows a comparison of concentrations of metal ion macronutrients inside and outside a typical mammalian cell [5].

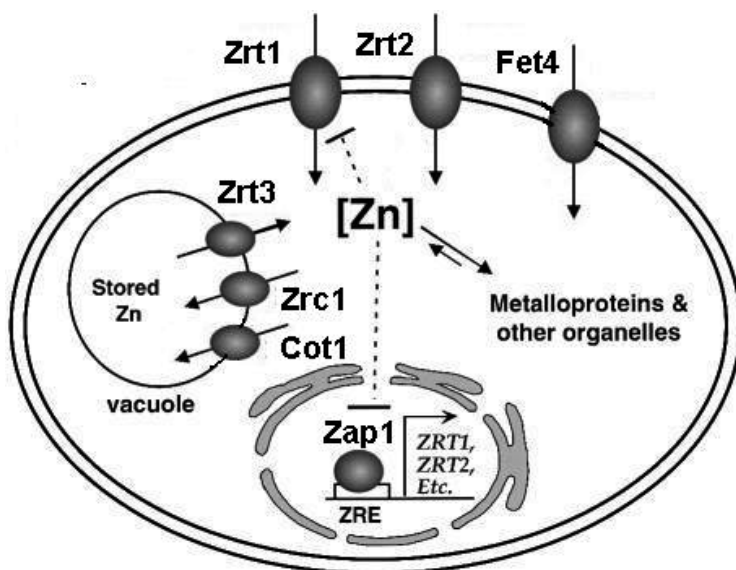
**Table 1.2: Comparison of concentration values of four major metal ions on the two sides of the plasma membrane of a typical mammalian cell [Taken from Ref. 5]**

Metal	Intracellular cytosolic concentration (mM)	Extracellular concentration (mM)
Na <sup>+</sup>	5-15	145
K <sup>+</sup>	140	5
Mg <sup>2+</sup>	0.5	1-2
Ca <sup>2+</sup>	10 <sup>-4</sup>	1-2

From this table, we can see that a typical mammalian cell maintains high gradients of Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> (especially the gradient of Ca<sup>2+</sup>) across the cell membrane. Such metal ion gradients are critical for the formation of membrane potential [126] and calcium signals.

#### 1.3.3.1 Various Proteins Involved in Metal Ion Homeostasis

Metal ion homeostasis process involves coordination between ion uptake, distribution, storage and efflux. All these relevant tasks are accomplished by proteins and genes with intricate interactions which can be illustrated by schematic graphs and complex networks. For example, the following schematic graph Fig. 1.2 describes the zinc homeostasis system in yeast [133]:



**Figure 1.2. A schematic graph depicting the zinc homeostasis system in *Saccharomyces cerevisiae* (Modified after Fig. 9 in Ref. 133).** Extracellular zinc ions are imported into the cell through Zrt1, Zrt2 and Fet4; the cytoplasmic zinc can be stored in metalloproteins, transported into the vacuole for storage through Zrc1 and Cot1 or transported to other organelles; the stored zinc in the vacuole can supplement the cytoplasmic zinc through Zrt3 when necessary; Zap1 is the critical regulatory protein which can sense the level of cytoplasmic zinc and activate a number of proteins including Zrt1, Zrt2, Zrt3, Fet4, Zrc1 and itself under zinc-limiting conditions<sup>3</sup>; excessive cytoplasmic zinc results in the inactivation of Zrt1 through endocytosis and vacuolar degradation; the cytoplasmic trafficking of zinc may involve chaperone-like proteins. ZRE: zinc-responsive elements.

In many cases, due to the evolution, the various proteins which accomplish a certain task in a specific metal ion homeostasis process in different organisms are homologous in both sequence and function. For example, an incomplete list of homologous proteins that play specific roles in calcium, copper and zinc homeostasis in bacteria, yeast, plant and human are shown in the following Table 1.3:

**Table 1.3: Homologous proteins that affect calcium, copper and zinc homeostasis in bacteria, yeast, plant and human [102, 246, 249]**

Metal	Bacteria	Yeast	Plant	Human	Description
Ca		Cch1	OsTPC1	CACNA1A	Voltage-dependent channel ( $\alpha 1A$ subunit)
		Pmc1		PMCA1a	$Ca^{2+}$ ATPase
		Pmr1		SPCA1	$Ca^{2+}$ ATPase
		Vcx1	CAX1,		$H^+/Ca^{2+}$ antiporter

<sup>3</sup> Actually the regulation of Zap1 on Zrt2 is complicated: Zrt2 is induced by Zap1 under conditions of mild zinc limitation but then repressed by Zap1 under more severe zinc-limitations.

			CAX5		
		Rcn1	RCN1	MCIP1, MCIP2, MCIP3	Calcineurin regulatory protein
		Mck1	AT5G14640	GSK3 $\beta$	Protein kinase
		Crz1		NFATc2	Calcineurin-dependent transcription factor
	CAL M_KL ULA	Cmd1	CAM6	CALML3	Ca <sup>2+</sup> sensor (CALML3 is Calmodulin-like protein 3; the rest three including CAM6, Cmd1 and CALM_KLULA are calmodulin proteins)
			CAM7	CAMI	Calmodulin (Ca <sup>2+</sup> sensor)
			CAM2	CAMII	Calmodulin (Ca <sup>2+</sup> sensor)
			CAM3	CALM3	Calmodulin (Ca <sup>2+</sup> sensor)
		Cna1		CNA1/CALN	Catalytic subunit of calcineurin (protein phosphatase)
		Cnb1		CNBII	Regulatory subunit of calcineurin (protein phosphatase)
Cu	CopA	Ctr1-Ctr3	COPT1	hCtr1/hCtr2	Copper transporter
		Atx1	CCH	Hah1p	Copper chaperone
	CopZ	Ces1	CCS1	CCS	Copper chaperone
		Cox17		hCox17	Copper chaperone
		Ccc2	RAN1, PAA1	ATP7A, ATP7B	Copper ATPase
		Sco1, Sco2		SCO1, SCO2	Copper-binding protein
	CueO	Fet3		Ceruloplasmin, Hephaestin	Multicopper oxidase
Zn		Zrt1-Zrt3	ZIP1-ZIP12	hZIP1-hZIP5	Zinc transporter
		Cot1		ZNT1	Zinc transporter
			ZAT1	ZNT2	Zinc transporter
			MTP4	ZNT4	Zinc transporter
			AT2G04620	ZNT7	Zinc transporter
		Zrc1		ZNT8	Zinc transporter
		Sod1	CSD1	hSOD1	Superoxide dismutase

Moreover, the same organism may possess homologous proteins functioning in the homeostasis systems of different metal ions. For example, *Bacillus subtilis* contains three homologous transcriptional regulator proteins (Fur, PerR, and Zur) which play central regulatory roles in iron and zinc homeostasis systems [149]. *E. coli* possesses two important homologous proteins (ZntR and Mer) which are critical metalloregulators in zinc homeostasis and mercury detoxification systems [182]. Another interesting phenomenon worthy of notice is that a specific kind of protein may play several roles in different metal ion homeostasis systems in a single organism. For example, Pmr1 in yeast is a high affinity P-type ATPase for transporting  $Mn^{2+}$  as well as  $Ca^{2+}$  into Golgi [246,249]. Sod1, CSD1 and hSOD1 function as superoxide dismutase in both iron and copper homeostasis systems in yeast, plant and human, respectively [249]. Metallothionein (MT) is a family of cysteine-rich proteins which functions as binding proteins for the buffering of both essential heavy metals such as zinc and copper and non-essential heavy metals such as cadmium, mercury and silver. Metallothionein proteins are widely found in all kinds of organisms including prokaryotes, yeast, plant and animals [249,251].

Finally, different organisms may use proteins which are homologous in function but not in sequence to play similar roles in specific metal ion homeostasis systems. For example, Zap1 and MTF1 are zinc-responsive transcription factors which function similarly (although they are not homologues in sequence) in zinc homeostasis systems in yeast and human [182]. Similar zinc-responsive transcription factors have been found in fungi, mammals, fish, and possibly plants, suggesting that the universal importance of transcriptional control of genes involved in zinc homeostasis [182].

### 1.3.3.2 Regulated Membrane Ion Transport

Regulated membrane ion transport constitutes the major scheme used by the biological cell to maintain optimal concentrations of metal ions in its cytosol. The lipid bilayer of cell membranes serves as a barrier to the passage of most polar molecules, including metal ions [5]. There are numerous ion transport proteins (including channels and transporters) on the cell membrane to transfer metal ions across the membrane. The regulations on membrane ion transport can be achieved by regulating the membrane concentration or the opening probability of relevant transport proteins.

The regulation on the membrane concentration (i.e., the number) of ion channels or transporters can be accomplished through transcriptional, translational, posttranslational or biochemical binding mechanisms. For example, in zinc homeostasis system in *E. coli*, the zinc influx through zinc transporter ZnuABC and the zinc efflux through zinc ATPase ZntA are regulated respectively by Zur and ZntR through transcriptional regulations [172]. In calcium homeostasis in yeast, the regulations of calcineurin on Pmc1 and Pmr1 through Crz1 are transcriptional whereas the regulation of calcineurin on Vcx1 is supposed to be posttranslational [50,51,132]. Another example of posttranslational regulation can be found in the zinc homeostasis system in yeast, where high intracellular zinc causes the inactivation of zinc transporter Zrt1 through endocytosis and the subsequent degradation of Zrt1 in the vacuole [133]. Regulation by biochemical binding

can be illustrated by the competitive inhibition on the availability of binding sites of transport proteins for a specific metal ion (e.g.,  $\text{Ca}^{2+}$ ) by other similar ions (e.g.,  $\text{Mg}^{2+}$ ).

Regulations on the opening probability of the ion transport proteins are even more common. For example, in mammalian calcium homeostasis systems, the opening probability of  $\text{IP}_3\text{R}$  proteins on the mammalian endoplasmic reticulum (ER) is regulated both by the cytosolic calcium concentration and  $\text{IP}_3$  concentration [57,69]. The opening probability of voltage-gated ion channels which play crucial role in excitable neuronal and muscle tissues is regulated by the membrane potential [69,105,154]. It has been reported that the opening probability of some L-type calcium channels are dynamically regulated by the calcium-bound calmodulin binding [243].

### 1.3.3.3 Other Strategies of Maintaining Homeostasis

Biological cells use a diverse range of strategies to maintain intracellular metal ion concentrations within an optimal range. Besides regulated membrane transport which is related with the regulations imposed on the metal ion uptake and efflux, cells also impose regulations on the storage and distribution processes to help achieve homeostasis [92,110,137,142,182,191,209]. These regulations can be realized through transcriptional, translational, post translational or biochemical binding mechanisms. For example, intracellular  $\text{Fe}^{2+}$  in *E. coli* represses (through Fur) the transcription of a small RNA (sRNA) named as RyhB, which facilitates the degradation of mRNAs encoding iron-using proteins [191]. This example exemplifies a combination of two control mechanisms: a transcriptional regulation on the expression of RyhB by Fur and a posttranscriptional regulation on the Fe-proteins by RhyB. It turns out later that sRNAs repressing iron-using proteins might be a quite common mechanism in bacteria because functional homologues of RhyB have been found in many other bacteria such as *P. aeruginosa* and *P. putida*. Regulations through translational mechanisms can be illustrated by the control of translation and RNA stability by iron-regulatory proteins in various organisms. For example, Cth2 is a zinc finger RNA-binding protein in yeast which regulates the mRNA stability of iron-using proteins in response to iron depletion [142].

An even more novel strategy to achieve homeostasis is to impose regulations on the regulatory proteins. The autoregulations of Zap1 in the yeast zinc homeostasis system and CopY (repressor) in the copper homeostasis system of *E. hirae* provide good examples [25,66,77,202]. However, there are more complicated cases. For example, in *E. coli*, ZntR is a metalloregulatory protein activating the zinc efflux ATPase ZntA. Recent experiments have shown that the binding of zinc to ZntR can reduce its degradation so that more ZntR molecules can be available for activating the zinc efflux pathway [172]. Another interesting example is that in yeast calcium homeostasis system, the activity of calcineurin is modulated by Rcn1 in a biphasic way (i.e., Rcn1 stimulates calcineurin at low concentrations and inhibits calcineurin at high concentrations) [100].

The great variety of possible mechanisms provides biological cells freedom of selecting suitable strategies to achieve homeostasis. It is quite common that a certain cell uses a combination of various strategies in a specific metal ion homeostasis system. For



example, as shown in Fig. 1.2, yeast cell uses both transcriptional control mechanisms to regulate membrane ion transport and the expression of the regulatory protein itself (through the transcriptional regulator Zap1) and posttranslational control mechanism to regulate the influx through Zrt1 to achieve zinc homeostasis. The iron homeostasis system in yeast exemplifies another good combination of several control mechanisms: Aft1 and Aft2 are iron-responsive transcription factors which activate the transcription of iron transport proteins and Cth2 during iron deprivation [142]. These transcriptional control mechanisms work together with the translational regulation of Cth2 on iron-using proteins to help the cell to maintain optimal iron concentration.

### 1.3.4 Metal Ion Signaling Systems

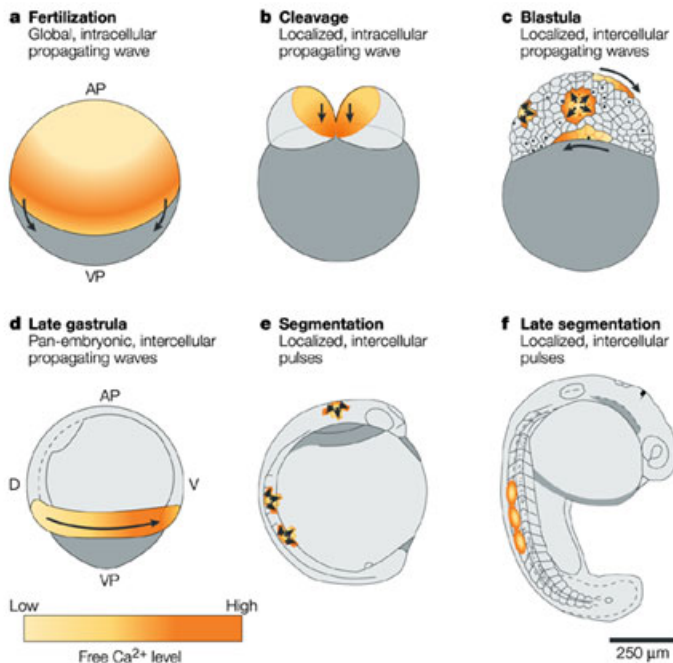
One of the basic characteristics of life is that living organisms can respond to stimuli. When a biological cell receives extracellular stimuli, it relies on diffusible signaling molecules named as intracellular second messengers (e.g.,  $\text{Ca}^{2+}$ ,  $\text{IP}_3$ , cAMP, NO, etc.) to activate effector proteins within the cell to exert a cellular response [251]. It has been well established for decades that  $\text{Ca}^{2+}$  is the most ubiquitous and versatile second messenger [20-22] whereas the identity of  $\text{Zn}^{2+}$  as a novel intracellular second messenger has been recently uncovered [238]. Moreover, the molecular identification of the extracellular calcium-sensing receptor (CaR) opens the possibility of that  $\text{Ca}^{2+}$  may function as a first messenger as well [106]. In the nervous system,  $\text{Zn}^{2+}$  is now regarded as neurotransmitter and functions as a transcellular and transmembrane signaling factor [219].

#### 1.3.4.1 Versatility and Universality of Calcium Signaling

The novelty of calcium signaling was firstly demonstrated by a “mistaken” experiment conducted by British clinician and pharmacologist Sidney Ringer (1836-1910) who used London tap water (instead of distilled water) containing calcium at nearly the same concentration as the blood to make a saline medium for suspending isolated rat hearts [39]. The beating of the hearts became progressively weaker and eventually stopped when the tap water was replaced by distilled water. Since then, the mysterious fog covering the iceberg of calcium signaling was gradually unveiled and now calcium is regarded as the most versatile carrier of signals regulating a myriad of important processes both inside and outside the cells [20-22,39,98,124,140].

The life of animals begins with a wave of calcium. When the sperm interacts with the egg,  $\text{Ca}^{2+}$  wave and subsequent sustained  $\text{Ca}^{2+}$  oscillations are initiated to trigger the developmental program (see Fig. 1.3a) [39,186]. During the embryogenesis,  $\text{Ca}^{2+}$  signaling controls the cleavage process (see Fig. 1.3b), contributes to body polarity and pattern formation and is critical for coordinating the motility of the cells. In later development, the differentiation of specific cell types (including muscle, neuron, heart and eyes, etc.) is again controlled by calcium (see Fig. 1.3c-d) [20-22,140,231]. In addition to playing critical roles in animal fertilization and development, calcium signaling is also important for the cell differentiation process and for the function of many different types of cells [20,126,138]. As shown by the experiment of Sidney Ringer,

calcium signals are required for cardiac muscle contraction. It has been recently found that calcium signaling controls the growth of heart as well [224]. In the nervous system, calcium signaling plays a pivotal role in receiving and transmitting neuronal signals, in controlling the release of neurotransmitters (e.g.,  $Zn^{2+}$ ), as well as in regulating excitability and the changes that underlie learning and memory [20-22]. Recent experimental discoveries have uncovered calcium as a central cell death regulator which triggers and modulates the apoptotic cell death process [20,162].



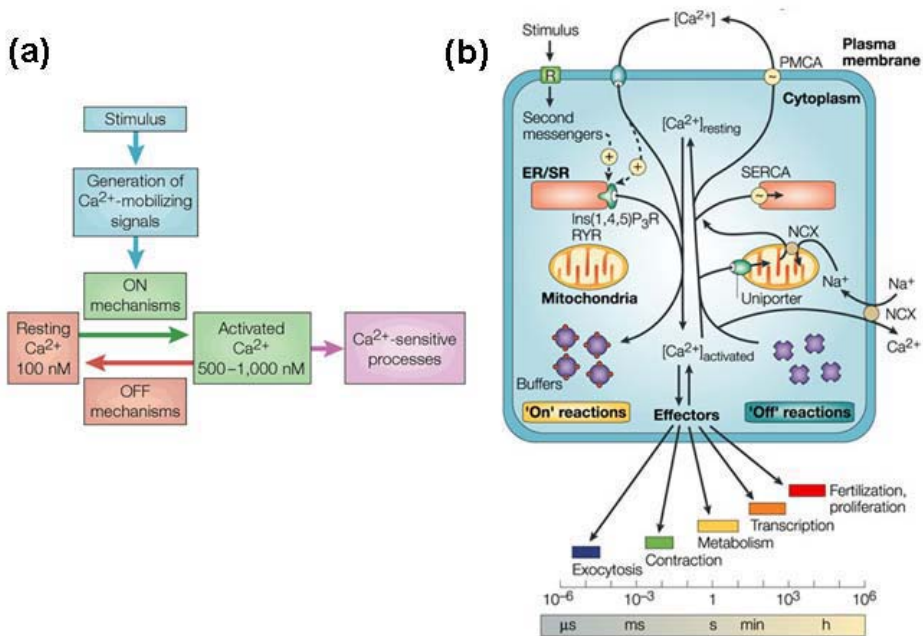
**Figure 1.3. Various forms of calcium signals in embryogenesis.** Please note the transition from a and b | intracellular, to c | localized intercellular, to d | pan-embryonic intercellular, and then back to e and f | localized intercellular  $Ca^{2+}$  signaling. AP: animal pole; D: dorsal; V: ventral; VP: vegetal pole (this figure is taken from Ref. 231).

In plants, calcium signals function in most aspects of growth and development including the response to drought, cold and salt stresses, mechanical wounding, symbionts and pathogens [94,184,185].  $Ca^{2+}$  signals are implicated in various responses to plant hormones (such as abscisic acid and auxin) and have been shown to play an essential role in pollen tube growth and fertilization. In yeast, as we will show in Chapter 2 and 3, there exists an elaborate calcium homeostasis/signaling system whose components (except  $H^+/Ca^{2+}$  antiporter) have all functionally retained in animal cells (see Table 1.3) [174].  $Ca^{2+}$  signals are critical in the response of yeast cell to various stimuli such as pheromone, hypertonic shock, hypotonic shock, etc [17,108,150,165].

Compared with its well-established signaling role in eukaryotes, the role of calcium in prokaryotes is more elusive [60]. Calcium ions have been found to be involved in the maintenance of bacterial cell structure, motility, transport and cell differentiation processes such as sporulation, heterocyst formation and fruiting body development. The characterization of calcium-binding proteins and the identification of other relevant factors suggest the possible existence of calcium signal transduction in bacteria [60].

#### **1.3.4.2 Intracellular Calcium Signaling**

Calcium homeostasis is the basis of calcium signaling. As mentioned before, a biological cell maintains an extremely high gradient of  $\text{Ca}^{2+}$  concentration across the cell membranes through the functioning of its calcium homeostasis system. Extracellular stimuli cause the change of the opening probability of various calcium transport proteins (mostly channels) on the membranes and results in sudden calcium influx into the cytosol due to the extremely high gradient. Calcium signaling depends on the increased levels of cytosolic  $\text{Ca}^{2+}$  concentrations derived either from sources outside the cell or within the organelles such as ER (in mammalian and plant cells) and/or the vacuole (in plant and yeast cells). A calcium signaling network consists of numerous components (the  $\text{Ca}^{2+}$  signaling toolkit) which can be grouped into four functional units as described in Fig. 1.4a [21-22].



**Figure 1.4. The Functional Units and Dynamics of Calcium Signaling.** (a) The four functional units (colored as blue, green, purple and pink, respectively) of the  $\text{Ca}^{2+}$  signaling network: (i) Extracellular stimuli triggers the signaling process by generating  $\text{Ca}^{2+}$ -mobilizing signals. (ii)  $\text{Ca}^{2+}$ -mobilizing signals activates the ON mechanisms through which cytosolic  $\text{Ca}^{2+}$  is fed. (iii) Numerous  $\text{Ca}^{2+}$ -sensitive processes are stimulated by  $\text{Ca}^{2+}$  signals (this graph is taken from Ref. 22). (iv) The resting state of cytosolic  $\text{Ca}^{2+}$  concentration is restored through the functioning of the OFF mechanisms. (b) Calcium signaling dynamics and homeostasis in animal cells (this graph is taken from Ref. 21).

A more detailed schematic graph depicting the dynamical process of calcium signaling and homeostasis in animal cells is shown in Fig. 1.4b. From the top left corner of this graph, we can see that extracellular stimulus acts on the receptor protein and induces both the entry of external  $\text{Ca}^{2+}$  through calcium channels and the formation of second messengers such as  $\text{IP}_3$  molecules which will act as  $\text{Ca}^{2+}$ -mobilizing signaling molecules to activate the  $\text{Ins}(1,4,5)\text{P}_3\text{R}$  channels and the ryanodine receptor (RYP) on the ER membrane. The opening of channels on both plasma membrane and ER membrane constitutes the 'On' mechanisms which result in the rise of cytosolic calcium level with the occurrence of the 'On' reactions. Most of the rushed-in cytosolic  $\text{Ca}^{2+}$  (shown as red dots) is bound to buffers, whereas a small proportion binds to the effectors that activate various cellular processes that operate over a wide temporal spectrum. The OFF mechanisms (see Fig. 1.4a) are composed of pumps and exchangers including the sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA), the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) and the plasma-membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) and Uniporter on the membrane of

mitochondria which sequester  $\text{Ca}^{2+}$  into the organelles or outside the cell and promote the recovery of the resting state<sup>4</sup> [21-22].

### 1.3.4.3 Extracellular Calcium Signaling

The well-established identity of calcium as the most versatile second messenger has vastly eclipsed its role as a first messenger. However, it seems much more natural if we consider the possible toxicity of calcium. The necessity of maintaining calcium homeostasis in the extracellular body fluid requires a detector to detect the extracellular calcium concentration and this role is played by CaR, a G-protein-coupled receptor which has now been found to be expressed in all major organs such as brains, pancreas, blood vessels, parathyroid gland, thyroid gland and heart [106,200,207]. CaR functions in the parathyroid and thyroid glands to tightly regulate blood calcium levels through regulating the secretion levels of parathormone (i.e., PTH, a hormone which stimulates calcium release from bone, calcium absorption by the intestine and reabsorption by the kidney) and calcitonin which can antagonize the effects of PTH [68,106]. It has been suggested that the CaR (also named as CaSR) may function in the adult brain by modifying the architecture of dendrites in response to changes in synaptic activity [227]. The discovery of CaR in key components of the cardiovascular system indicates that the CaR may modulate myogenic tone (i.e., pressure-induced constriction) through activation of nitrogen oxide (NO) production and K-channels in the vascular tree, thus making calcium a first messenger that modulates the system [200]. It has been experimentally shown *in vitro* that an intracellular calcium signal may induce generation of an extracellular calcium signal that is sensed by the CaR on neighbouring cells or even on the same cell [200]. Such novel mechanism provides an alternative strategy for the propagation of calcium signals across the cells other than using gap junctions as found in neuronal cells and may underlie the formation of localized intercellular calcium signals in embryogenesis and development (see Fig. 1.3).

### 1.3.4.4 Calcium Signaling Toolkit

The incredible versatility of calcium signaling arises through the use of an extensive  $\text{Ca}^{2+}$ -signalling toolkit composed of receptors, transducers, channels, pumps and exchangers, calcium buffers, calcium effectors, calcium-sensitive enzymes and processes [21-22,94]. For example, according to Harper *et al.* (2005) [94], *Arabidopsis thaliana* (a model plant) is predicted to have a large number of calcium transport proteins (including 20 cyclic nucleotide-gated channels, 30 glutamate receptors, a single two-pore  $\text{Ca}^{2+}$  channel, 14  $\text{Ca}^{2+}$  pumps and 12 potential  $\text{H}^+/\text{Ca}^{2+}$  exchangers) and a large number of  $\text{Ca}^{2+}$  effector proteins (including 9 calmodulins, ~50 calmodulin-like proteins, 7 annexins and 10 calcineurin B-like proteins).

The signaling toolkits in different organisms may not only share many features as partially shown in Table 1.3), but also have important differences. For example, in mammalian cells, there are hundreds of G-protein-coupled receptors (including

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<sup>4</sup> Please note that during the 'off' reactions, the rapidly sequestered  $\text{Ca}^{2+}$  by mitochondrial uniporter is released more slowly back to the cytosol by  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) on the mitochondrial membrane.

bradykinin receptors B<sub>1</sub>, B<sub>2</sub> and CaR) and 58 known tyrosine-kinase-linked receptors whereas *A. thaliana* has no tyrosine-kinase-linked receptors and only one identified (GCR2) and one putative (GCR1) G-protein-coupled receptor. Unlike animals, land plants in general do not have protein kinase C, stereotypical calmodulin dependent protein kinases (CaMKs) or calcineurin (see Table 1.3). Instead, they have Ca<sup>2+</sup>-dependent protein kinases (CDPKs), chimeric Ca<sup>2+</sup> and calmodulin-dependent protein kinases (CCaMKs) and CDPK-related kinases (CRKs), etc., some of which are regulated by Ca<sup>2+</sup>-binding proteins [94].

### 1.3.4.5 Spatial and Temporal Aspects of Calcium Signaling

As mentioned before, repetitive intracellular waves of calcium trigger the developmental program of animals. In animals and plants, calcium signals may appear in various forms such as intracellular localized signals (blips, quarks, puffs and sparks) and intercellular or intracellular global signals (transients, pulses, oscillations and waves) [20-22,231]. For example, the schematic graph shown in Fig. 1.3 describes the different forms of calcium signals used in six developmental stages of a representative embryo (e.g., a zebrafish) [231].

The spatial organization of calcium signaling toolkit (especially channels) may have influence on the formation of various forms of Ca<sup>2+</sup> signaling. Intracellular Ca<sup>2+</sup> blips, quarks, puffs, or sparks represent elementary Ca<sup>2+</sup> signaling units which are thought to reflect the local opening of individual (or small groups of) Ca<sup>2+</sup>-release channels on the ER membrane. Sufficiently strong and persistent extracellular stimulus may result in regenerative Ca<sup>2+</sup> wave across the cytoplasm due to the propagation of the localized signals. Single Ca<sup>2+</sup> wave results in pulses and transients whereas repetitive Ca<sup>2+</sup> waves lead to oscillations. It is believed that both the waves and the oscillations depend, in part at least, on a combination of positive and negative feedback regulations by cytosolic Ca<sup>2+</sup> on the calcium-releasing channels on ER (IP<sub>3</sub>Rs and RyRs): the released Ca<sup>2+</sup> initially stimulates more Ca<sup>2+</sup> release, a process known as Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR), whereas later, high cytosolic Ca<sup>2+</sup> inhibits further release [20-22].

As shown in Fig. 1.4b, the processes regulated by calcium signaling operate over a wide temporal spectrum. In the case of fast signaling events such as synaptic transmission or cardiac contraction, the effector systems respond to calcium signals within μs to ms range whereas in the case of slower events (e.g., oocyte activation at fertilization), calcium signals (the repetitive calcium waves in the case of fertilization) may last as long as several hours to fully accomplish their tasks [21].

### 1.3.4.6 Calcium Signature Hypothesis vs. Chemical Switch Hypothesis

The central question in the field of calcium signaling is related with its specificity. How can calcium signaling link different extracellular signals to a myriad of cellular responses?

Calcium signature hypothesis assumes that calcium signals are encoded in the temporal and spatial nature and the amplitude of cytosolic calcium concentration changes (the

calcium signature), which are later decoded by the effectors [20,169,217]. It has been experimentally demonstrated that in mammalian cells,  $\text{Ca}^{2+}$  signals varying in amplitude, duration, frequency, and localization may result in differential responses. For example, changes in the amplitude and duration of a  $\text{Ca}^{2+}$  signal in B lymphocytes can induce differential activation of transcription factors [59]. Experiments conducted by Li *et al.* (1998) demonstrated that the degree of gene expression can be determined by the frequency of  $\text{Ca}^{2+}$  spikes via nuclear factor of activated T-cell (NFAT) [128]. It has been experimentally shown that  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) are able to decode frequency-modulated  $\text{Ca}^{2+}$  signals which widely exist in many cells including hepatocytes, salivary glands, endothelial cells and smooth muscle cells. In wildtype *Arabidopsis* guard cells, Allen *et al.* (2000) demonstrated that calcium signature (in the form of oscillations) is produced in response to extracellular calcium and  $\text{H}_2\text{O}_2$  to specifically direct stomatal closure and such signature can be mimicked by artificially produced calcium oscillations [8,9].

In plant calcium signaling field, however, the dogma of calcium signature has aroused many doubts and debates [170,189]. For example, Scrase-Field *et al.* (2003) argued that calcium may be merely an essential chemical switch in signaling and that calcium signature hypothesis describes the exception (e.g., in the just mentioned guard-cell signaling) rather than the rule. They enumerated experimental evidence suggesting that signal specificity can be the responsibility of some signaling components other than calcium [189].

#### 1.3.4.7 Zinc Signaling

The very recent exciting discovery of the existence of intracellular zinc wave by Yamasaki *et al.* (2007) has provided evidence showing that zinc is a novel intracellular second messenger [238]. Their experiments demonstrated that a zinc wave was induced several minutes after mast cells were stimulated by an extracellular stimulus called as  $\text{Fc}\epsilon\text{RI}$  cross-linking. This zinc wave originated from the perinuclear region that includes the ER and was dependent on both calcium influx and MAPK/ERK kinase (MEK) activation. The role of the zinc wave is supposed to be to regulate the duration of MAPK activation, inhibit tyrosine phosphatase activity and modulate signaling quantity of the relevant events [238].

Similar as  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$  has been implicated as an important messenger in a number of cellular signaling pathways in animals, including those involved in synaptic transmission, cell proliferation and apoptosis. It has been noticed for decades that  $\text{Zn}^{2+}$  plays some enigmatic role in the central nervous system. Experimental observation has shown that  $\text{Zn}^{2+}$  is accumulated by specific neurons into synaptic vesicles and can be released by stimulation in a  $\text{Ca}^{2+}$ -dependent manner [62,219]. Now  $\text{Zn}^{2+}$  is regarded as a neurotransmitter modulating both excitatory and inhibitory neurotransmission. The very recent hypothesis about the existence of a specific zinc-sensing receptor (ZnR) may explain some interesting phenomena, for instance, the application of  $\text{Zn}^{2+}$  to epithelial cells in culture or to PC-3 (Human prostate cancer cell line) cells leads to an increase in intracellular  $\text{Ca}^{2+}$  concentration via the  $\text{IP}_3$  pathway [62,97]. The molecular identification

of ZnR in the future will eventually establish zinc's identity of a first messenger. Because extracellular  $Zn^{2+}$  promotes cell growth in numerous cancer cell types (e.g., HT-29, PC-3 and NIH3T3 fibroblasts) by activating and regulating major signaling pathways (e.g., MAP kinase pathway), the thorough understanding of zinc signaling in these cells will help devise therapeutic strategies to attenuate tumor growth and cure related diseases [62].

## 1.4 Feedback Control Theory and Network Motifs

The biological cell is a collection of protein machines full of feedback regulations. The mysterious ability that a biological cell can keep a relatively stable internal environment in an ever-shifting extracellular environment relies on a variety of negative and positive feedback controls embedded in its complicated intracellular networks composed of proteins, genes and metabolites [5,32,74,213,220]. Negative feedback controls constitute the central scheme of metal ion homeostasis [241]. For example, *E. coli* cell employs transcriptional negative feedback control mechanisms to regulate the uptake and efflux of zinc ions whereas in yeast cells [172], the negative feedback control pathway through calcineurin plays an important role regulating the long-term cytosolic calcium homeostasis [132].

### 1.4.1 A Bit of History of Feedback Control

The primary motivation for feedback control in ancient time was the need for the accurate determination of time. As early as about 270BC, the Greek inventor and mathematician Ktesibios (285-222BC) invented an improved water clock (a clepsydra) operated by means of a regulated flow of water into a tank where the water level is measured by a float and fed back for the regulation of the inflow. Thus the water level in the tank is kept as a constant which causes a constant flow through a tube at the bottom into a second tank whose water level can be measured to determine the time elapsed [220,251].

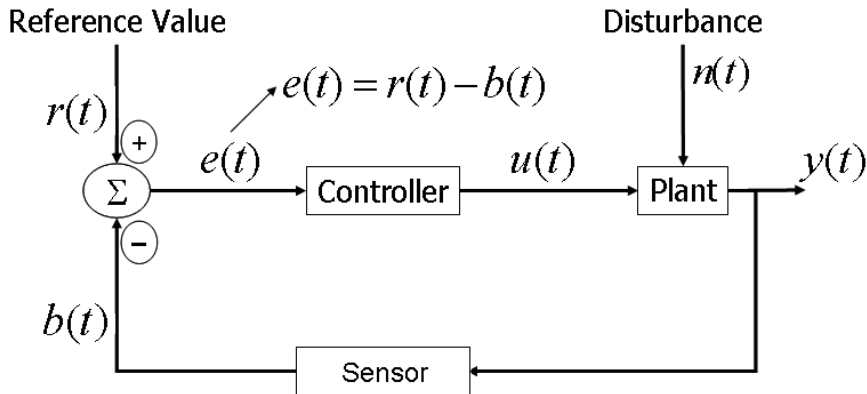
The concept of feedback was later used by Charles Darwin (1805-1882) who devised the theory that feedback over long time periods is responsible for the evolution of species. Vito Volterra (1860-1940) used this concept to explain the balance between two populations of fish in a closed pond [251]. However, the most influential person in the history of feedback control theory is Norbert Wiener (1885-1964) who introduced the fruitful concepts of positive and negative feedbacks in biology and founded the theory of cybernetics [233]. It is very worth to mention that Wiener's work owed much to Walter Cannon (1871-1945) who developed the concept of homeostasis and popularized it in his book "*The Wisdom of the Body*" [38]. Cannon noticed the remarkable stability of numerous blood properties in mammals in the face of enormous environmental change and termed this process as homeostasis.

### 1.4.2 Basic Feedback Control System

As shown in Fig. 1.5, the most elementary control system consists of three components: a plant (the object to be controlled), a sensor and a controller. The output of the plant (i.e.,



$y(t)$ ) is measured by the sensor whose output (i.e.,  $b(t)$ ) is compared with a reference input (i.e.,  $r(t)$ ) and then the deviation (i.e.,  $e(t)$ ) is fed back to the plant through a controller [61,127]. However, in many complex feedback systems as those considered in this thesis, the reference signal is usually hidden or even absent.



**Figure 1.5. Elementary control system.**  $y(t)$  : the plant output and measured signal;  $u(t)$  : the plant input (i.e., actuating signal);  $n(t)$  : the external disturbance;  $b(t)$  : sensor output;  $r(t)$  : reference or command input;  $e(t)$  : error or deviation (this figure is based on Ref. 61).

One of the first mathematical analysis of control systems was the frequency-domain approach which is based on Laplace transformation.

$$\mathbf{L}[f(t)] = F(s) = \int_0^{\infty} f(t)e^{-st} dt \quad (1.1)$$

The central concept of frequency-domain approach is that of a *transfer function* (defined as  $Y(s)/U(s)$  where  $Y(s)$  and  $U(s)$  denote the Laplace transform of the output and the input of the plant, respectively) which turns out to embody the *transfer characteristics* of the system. However, this approach is appropriate for linear time-invariant systems, especially for single-input/single-output systems where the graphical techniques are very efficient [61,251]. As we will see later, most control systems considered in this thesis are non-linear and time-variant, so we will not discuss too much the frequency-domain approach here.

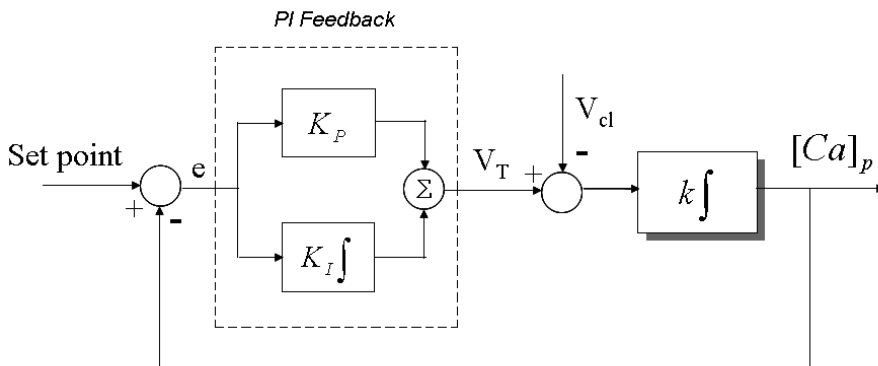
### 1.4.3 Negative Feedback

Negative feedback is the basic mechanism by which systems, whether mechanical, electrical, or biological, maintain their equilibrium or homeostasis. In his famous book “Cybernetics”, Norbert Wiener defined negative feedback as “the feedback (which) tends to oppose what the system is already doing” [233]. In another word, negative feedback

counteracts the effect of the stimulus, thus stabilizes outputs and enables biological systems to operate with resilience.

In many other cases, negative feedback can also result in oscillations. Norbert Wiener discussed in detail how a simple linear negative feedback can result in an oscillation whose amplitude does not increase or even an unrestrained and increasing oscillation (i.e., catastrophe) [233]. Negative feedback has been proposed to be the underlying basic mechanism responsible for various oscillations found in protein synthesis, MPF activity, MAPK signaling pathways, and circadian rhythms [82,221]. However, there are more complex cases in which oscillations arise in systems containing both positive and negative feedbacks [114,188,139].

The blood calcium homeostasis model proposed by EL-Samad *et al.* (2002) gives a nice example of negative (so called proportional plus integral (PI)) feedback control, the block diagram of which is shown in Fig. 1.6 [68].



**Figure 1.6. A block diagram of the blood calcium homeostasis model.** This assumes that the studied system employs a PI (proportional + integral feedback) control mechanism.  $[Ca]_p$ : plasma calcium concentration;  $V_T$ : the total rate of calcium introduced into the plasma;  $V_{cl}$ : the calcium clearance rate from the plasma;  $k$ : the inverse of the total plasma volume;  $K_P$ : the real constant of the proportional feedback control;  $K_I$ : the real constant of the integral feedback control;  $e$ : the error; set point: the desired value of  $[Ca]_p$  (this figure is based on Ref. 68).

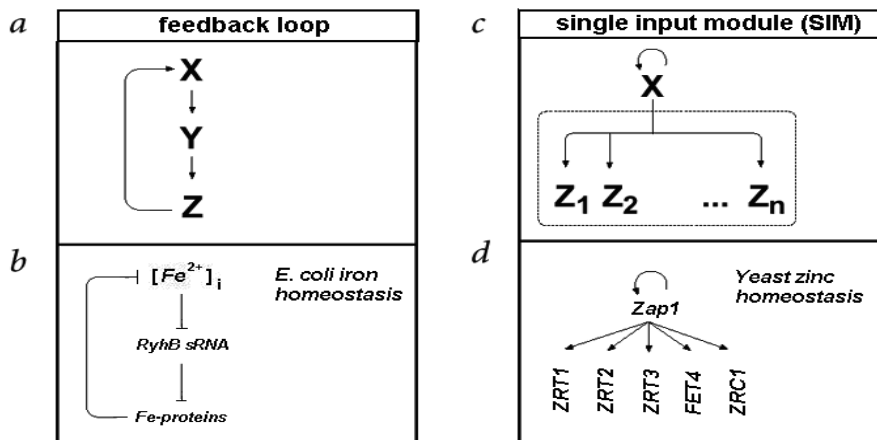
For more details on the physiological basis and the mathematical expression of this model, please see the original paper [68].

#### 1.4.4 Network Motifs

Networks arise naturally in biology including gene regulation networks, protein networks, epidemiology networks, ecological food webs, etc [10,11]. In biological cells, metal ion homeostasis and signaling processes usually involve complex networks composed of genes, proteins and metabolites. For example, it is estimated that the calcium signaling

network in mammalian cardiac myocyte consists of tens to hundreds of components [224], part of which related to cardiac hypertrophy is described in Chapter 4 of this thesis. To uncover the design principle of such complex networks and understand their dynamics, a natural idea is to break them down into basic building blocks which are termed as 'network motifs' and study the specific functions of these motifs by using mathematical models [10,11,193].

Network motifs are defined as “patterns of interconnections that recur in many different parts of a network at frequencies much higher than those found in randomized networks” [10,193]. Since their first definition, a number of network motifs such as feedforward loop motif, autoregulation motif, feedback loop motif, single input module (SIM) motif and coupled feedback loop motifs have been found in sensory transcription networks, developmental networks, signal transduction networks and neuronal networks [10,114,115,193]. As we will see in Chapter 6 of this thesis, similar motifs also exist widely in metal ion homeostasis and signaling networks, two examples of which are shown in Fig. 1.7.



**Figure 1.7. Two examples of network motifs.** (a). In feedback loop motif, component X regulates component Y, Y regulate Z and Z regulates X. (b). An example of (negative) feedback loop motif found in *E. coli* iron homeostasis system. Cytoplasmic  $Fe^{2+}$  (i.e.,  $[Fe^{2+}]_i$ ) in *E. coli* represses (through Fur) a small RNA (sRNA) named as RyhB, which facilitates the degradation of mRNAs encoding Fe-using proteins (i.e., Fe-proteins) [142,191]. (c). In SIM motif, a single component X regulates a set of components  $Z_1, Z_2, \dots, Z_n$ . X is usually autoregulatory. (d) An example of SIM motif found in yeast zinc homeostasis system. Zap1 is a zinc-responsive activator in yeast which activates the transcription of genes encoding cytosolic zinc influx transporters (ZRT1, ZRT2, ZRT3, FET4) and efflux transporter (ZRC1) (see Fig. 1.2).

## 1.5 Enzyme Kinetics and Nonlinear ODE Modeling

An enzyme is a substance that acts as a catalyst for chemical reactions. Enzymes reduce the needed activation energy of the reactions in living systems so that they can proceed at

rates that are useful to the cell [5,58,70,251]. In metal ion homeostasis and signaling systems, there are many enzyme catalyzed reactions. For example, the metal ion uptake and efflux processes are mediated by ion channels and transporters which can be regarded as enzymes. Calcineurin, which plays central role in calcium signaling in many organisms, is a special enzyme (phosphatase) which catalyzes the dephosphorylation of proteins such as Crz1 in yeast and NFAT in cardiac myocytes [208,224]. Thus basic knowledge about enzyme kinetics is quite necessary for modeling metal ion homeostasis and signaling systems.

### 1.5.1 Michaelis-Menten Kinetics

The kinetics of many enzyme-catalyzed reactions in solution has been proven to conform to the classic model of Michaelis and Menten as follows [58,178]:



Where in the first reaction, the enzyme ( $E$ ) and the substrate ( $S$ ) reversibly form an intermediate complex ( $ES$ ), which produces the product ( $P$ ) and releases the enzyme ( $E$ ) in the subsequent second reaction.  $k_1$  and  $k_{-1}$  denote the forward and the backward rate constants of the first reaction, respectively whereas  $k_2$  denotes the rate constant of the second reaction which is assumed as irreversible.

This model can be expressed into 4 ODEs and we can further simplify the system by making the quasi-steady-state hypothesis (i.e., we assume that the concentration of  $ES$  is steady), the velocity of the enzyme reactions (denoted as  $v$ ) can be calculated as follows:

$$v = \frac{V_{\max} [S]}{[S] + K_M} \quad (1.3)$$

Where  $V_{\max}$  denotes the maximal velocity of the production,  $[S]$  denotes the concentration of substrate and  $K_M$  denotes the Michaelis constant with dimensionality unit:  $\text{mol l}^{-1}$  (the concentration of substrate that leads to half-maximal velocity) which can be calculated as  $\frac{k_2 + k_{-1}}{k_1}$ .

### 1.5.2 Reversible Competitive Inhibition

The rate of an enzyme-catalyzed reaction can be reduced by inhibitors. Loss of enzyme activity may be either reversible, where activity may be restored by the removal of the inhibitor, or irreversible, where the loss of activity is time dependent and cannot be recovered during the timescale of interest [58]. In metal ion homeostasis and signaling

systems, reversible competitive inhibition happens quite frequently. For example, different metals such as ( $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ ) can compete for the binding of the same channel or transporter protein. Moreover, enzymes such as calcineurin usually have many target proteins which compete with each other for its binding. So some knowledge about reversible competitive inhibition is also very necessary.

Let us consider the case where two substrates  $S_1$  and  $S_2$  compete for the binding of the same enzyme  $E$  to produce different products ( $P_1$  and  $P_2$ ) as modeled by the following scheme:



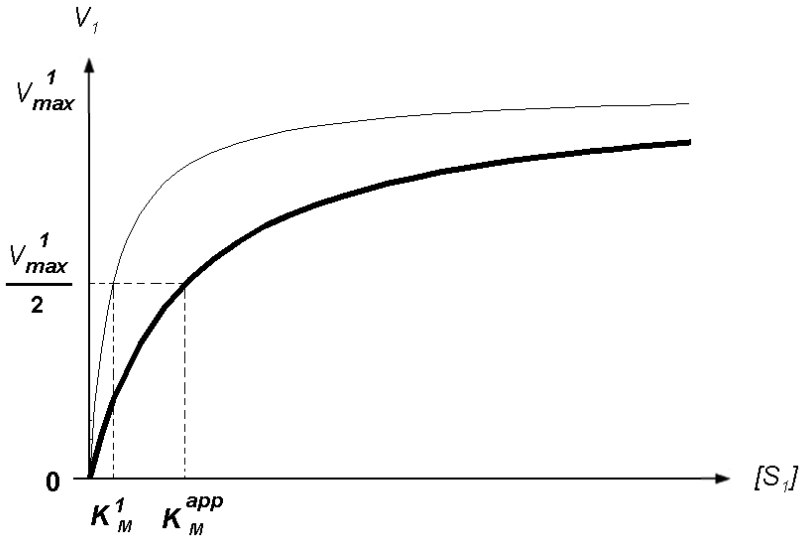
If we assume that the rates of product formation are much slower than attainment of the equilibrium (i.e.  $k_2 \ll k_{-1}$  and  $k_4 \ll k_{-3}$ ), the rates of formation of  $P_1$  and  $P_2$  are given by

$$v_1 = \frac{V_{\max}^1 [S_1]}{K_M^1 (1 + \frac{[S_2]}{K_M^2}) + [S_1]} \quad (1.6)$$

and

$$v_2 = \frac{V_{\max}^2 [S_2]}{K_M^2 (1 + \frac{[S_1]}{K_M^1}) + [S_2]} \quad (1.7)$$

, respectively where  $V_{\max}^1$  and  $V_{\max}^2$  denote the maximal velocity of the production of  $P_1$  and  $P_2$ .  $K_M^1$  denotes the Michaelis constant of  $S_1$  which is called the inhibition constant of  $S_1$  over  $S_2$ . Similarly,  $K_M^2$  denotes the Michaelis constant of  $S_2$  which is also called the inhibition constant of  $S_2$  over  $S_1$ . The effect of competitive inhibition on the velocity of the enzyme reaction is illustrated in Fig. 1.8.



**Figure 1.8. Effect of enzyme competitive inhibition.** The solid curves depict the production velocity of  $P_1$  as a function of the concentration of  $S_1$  without inhibition (thin solid curve) and with inhibition (thick solid curve) from  $S_2$ , respectively. Without inhibition, the half-maximal velocity is reached when  $[S_1] = K_M^1$  whereas with competitive inhibition from  $S_2$ , the half-maximal velocity is reached only when  $[S_1] = K_M^{app} = K_M^1 \left(1 + \frac{[S_2]}{K_M^2}\right)$  where  $K_M^{app}$  denotes the apparent Michaelis constant of  $S_1$ .

### 1.5.3 Nonlinear ODE Modeling

As mentioned before, to a first approximation, ODEs provide a good framework for modeling the dynamics of biochemical reaction networks (see Section 1.2.2) [120,213]. The immense variety of nonlinearities in living systems constitutes one of the major difficulties for modeling biological systems [113,139,141,213]. As we will see in later chapters, all the models presented in this thesis are nonlinear ODEs. In the case of intracellular metal ion homeostasis and signaling systems, the nonlinearity mainly arises from several sides:

- (i). The nonlinear characteristics of the extracellular stimulus. For example, a very universal stimulus for the perturbation of metal ion homeostasis system is the step rise of extracellular ion concentration which is nonlinear.
- (ii). The nonlinear interaction between the extracellular stimulus and the cell. For example, in Chapter 3, we can see that extracellular hypertonic shock can lead to the nonlinear evolution of the yeast cell volume.

(iii). The nonlinear transport characteristics of ion channels and transporters. For example, the uptake behavior of many metal ion transporters (and channels) can be modeled by the Michaelis-Menten kinetics which is essentially nonlinear.

(iv). The nonlinear interaction among proteins, genes and metabolites. For example, according to law of mass action, a reversible biochemical reaction ( $A + B \rightleftharpoons C$ , which means reactant  $A$  binds with reactant  $B$  to form product  $C$ ) can be described by the following ODEs:

$$\frac{dC}{dt} = -\frac{dA}{dt} = -\frac{dB}{dt} = -r_b C + r_f AB \quad (1.8)$$

which naturally contain a nonlinear term  $AB$  (in the above equations,  $r_f$  and  $r_b$  denote the forward and backward rate constants, respectively). Similar nonlinearity arises in metal ion sensing process because of the binding of sensor proteins with the metal ions.

Nonlinear ODEs systems may have steady or unsteady (oscillating, chaotic, etc.) solutions [218]. In metal ion homeostasis systems, the steady state value of the variable in the ODEs solution denoting the regulated metal ion concentration is the most important because the task of all the components of the ion homeostasis system is just to keep this concentration value within an optimal range with no regard to various physiologically reasonable perturbations [123]. In the case of metal ion signaling systems, oscillating solutions can become quite important because of the widely existing phenomena of oscillations such as calcium oscillation and heart beating related to metal ion signaling [69,221]. Chaotic solutions may be useful (although rarely compared with steady and oscillating solutions) to mimic the abnormal behavior in pathological conditions, for instance, the cardiac arrhythmia.

## 1.6 Thesis Overview

The central question of this thesis is how to use mathematical models to simulate the complicated dynamics arising from metal ion ( $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ ) homeostasis and signaling systems in various organisms and to explain certain mutant behavior. First we will present a preliminary mathematical model which we developed for yeast calcium homeostasis, a work where all experimental data and some critical parameters are taken from literature (see Chapter 2). This concise model consists of 4 ODEs (ordinary differential equations) and is based on an assumption of quick feedback regulation on cytosolic  $\text{Ca}^{2+}$  sequestering transporters (Pmc1, Pmr1, etc.) through a gene expression feedback pathway through calmodulin (a ubiquitous  $\text{Ca}^{2+}$  sensor) and calcineurin ( $\text{Ca}^{2+}$  phosphatase). Simulation results show that our model can qualitatively reproduce the experimentally observed response curve of real yeast cell responding to step-like disturbance in extracellular  $\text{Ca}^{2+}$  concentration and correctly predict certain mutant behavior.

After the publication of this first model, we managed to establish good collaboration with Professor Kyle W. Cunningham of the Johns Hopkins University. We designed some new experiments which showed that the afore mentioned calcineurin-dependent expression feedback pathway has little or no effect on aequorin luminescence traces within the first few minutes of a  $\text{Ca}^{2+}$  shock. Therefore we built a new mathematical model that omits calcineurin-dependent feedback and instead includes rapid  $\text{Ca}^{2+}$ -dependent feedback inhibition of  $\text{Ca}^{2+}$  influx pathways which fit well new experimental data (see Chapter 3). Our simulation results strongly suggest the existence of a new calcium transporter M on the *yvc1 cchl* plasma membrane under hypertonic shock.

Next we move from a relatively simple organism (yeast) to a more complex organism (mice). Due to Professor Kyle W. Cunningham's suggestions, we have made detailed investigations into the possible mechanisms of the paradoxical, dual role of MCIP1 (Modulatory calcineurin-interacting protein 1) in mice cardiac hypertrophy. By including some recent experimental findings, we constructed a mathematical model composed of 28 ODEs to describe the complex underlying calcium-calcineurin signaling network (see Chapter 4). This complicated model can correctly predict the mutant (MCIP1<sup>-/-</sup>) behavior under different stress such as PO (pressure overload) and  $\text{CaN}^*$  (activated calcineurin) overexpression.

Next we proceed to zinc homeostasis, although this time we study an even simpler organism - *E. coli*. The zinc homeostasis in *E. coli* involves a highly symmetrical structure which consists of repression on zinc influx through ZnuABC transporter by Zur ( $\text{Zn}^{2+}$  uptake regulator) and activation on zinc efflux via ZntA by ZntR (a zinc-responsive regulator). We constructed a mathematical model composed of 14 reactions which can quantitatively reproduce and interpret various reported results of the *in vitro* experiments of  $\text{Zn}^{2+}$  homeostasis system in *E. coli* (see Chapter 5) such as Zur-DNA binding curve, Zur and ZntR sensitivity curves, etc.

We finish the investigations on particular homeostasis/signaling networks and try to discover the general principles underlying metal ion homeostasis and signaling networks. We enumerate network motifs found in these systems such as the autoregulation motif, SIM (single-input module) motif, signaling cycle motif and coupled feedback motifs (see Chapter 6) and show the great variety of edge relations in those motifs. Particularly, we develop an ODE model and present simulation results to show the modulation on the properties of the signaling cycle motif by an inhibitor.

In the concluding Chapter 7, we summarize this thesis, discuss several interesting issues related to metal ion homeostasis and signaling systems based on our findings and indicate the directions of future work.