Mathematical modeling of metal ion homeostasis and signaling systems

Cui, J.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
Chapter 6  Network Motifs and Their Functions

6.1  Introduction

Networks arise naturally in biology for describing the intricate interactions of biological entities. To uncover the design principle of dauntingly complex networks and understand their dynamics, Uri Alon and his colleagues (2002) defined “network motifs” which can be thought of as recurring circuits of interactions and regarded as the building blocks of complex networks [193]. Just as each door or window, which is the building block of normal buildings, has its specific structure and functions, each network motif has its specific structure and is believed to carry out specific information-processing functions. Since these networks motifs are typically small size circuits of interactions and are relatively easy to analyze using mathematical models, the understanding of the specific functions of network motifs has been believed by Alon et al. and many other people to be a nice way to help illuminate the dynamics of much more complex systems in which each motif appears [10,11,115].

Network motifs were first systematically defined in the transcription network of *E. coli* and have later been found in organisms from bacteria and yeast to plants and animals. An incomplete summary of network motifs and their functions is shown in Table 6.1.

**Table 6.1: Network motifs and their functions.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Pattern</th>
<th>Functions</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auto-regulation</td>
<td>Positive autoregulation (PAR)</td>
<td><img src="" alt="Positive Autoregulation" /></td>
<td>In transcription networks, PAR slows down the response time of gene circuits and can usually enhance cell-cell variation [10,11].</td>
</tr>
<tr>
<td>Negative autoregulation (NAR)</td>
<td><img src="" alt="Negative Autoregulation" /></td>
<td>In transcription networks, NAR speeds up the response time of gene circuits and can reduce cell-cell variation.</td>
<td></td>
</tr>
</tbody>
</table>

25 This Chapter is based on: Jiangjun Cui and Jaap Kaandorp, The modulation on the properties of signaling cycle motif by an inhibitor (in preparation).
<table>
<thead>
<tr>
<th>Feed-Forward Loop (FFL)</th>
<th>Coherent type-1 FFL (C1-FFL)</th>
<th>In transcription networks, the C1-FFL is a 'sign-sensitive delay' element and a persistence detector [10].</th>
<th>Component X regulates component Y and both of them regulate Z.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incoherent type-1 FFL (I1-FFL)</td>
<td>In transcription networks, the I1-FFL is a pulse generator and response accelerator [10].</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Other FFLS**

**Single-Input Module (SIM)**

In transcription networks, SIM allows coordinated expression of a group of genes with shared function. It can generate a temporal expression program, with a defined order of activation of each of the target promoters [10,11,193].

Component X regulates a group of other components \(Z_1, Z_2, \ldots, Z_n\).

**Bifan**

Not well defined [109]

Component X regulates both Z and W. At the same time, component Y regulates both Z and W.

**Diamond**

Not totally defined

Both components Y and Z are regulated by X and at the same time,
<table>
<thead>
<tr>
<th>Feedback Loop with Two nodes</th>
<th>Positive feedback loops (PFL)</th>
<th>X ↔ Y</th>
<th>In signal transduction networks, PFL amplifies the signal and results in slower response. It can also lead to bistability and hysteresis [115].</th>
<th>Components X and Y regulate each other.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X ↔ Y</td>
<td></td>
<td>In PFL, both X and Y positively regulate each other.</td>
<td>In NFL, only one regulation is positive and the other one is negative.</td>
</tr>
<tr>
<td></td>
<td>X ↔ Y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative feedback loops(NFL)</td>
<td>X ↔ Y</td>
<td></td>
<td>NFL helps maintaining homeostasis and plays important role in signal adaptation or desensitization to sustained stimulation. It functions as noise filters and accelerates signal responses [115].</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X ↔ Y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coupled Feedback Loops</td>
<td>Two positive feedback loops (PP)</td>
<td>X ↔ Y ↔ Z</td>
<td>PP induces a slower but amplified signal response and enhances bistability [115].</td>
<td>Components X and Y regulate each other. At the same time, Y and Z regulate each other.</td>
</tr>
<tr>
<td></td>
<td>X ↔ Y ↔ Z</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A positive feedback loop and a negative feedback loop (PN)</td>
<td>X ↔ Y ↔ Z</td>
<td>PN guarantees reliable decision-making by properly modulating signal responses and effectively dealing with noise [114,115].</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X ↔ Y ↔ Z</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X ↔ Y ↔ Z</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Two negative feedback loops (NN)</td>
<td>X ↔ Y ↔ Z</td>
<td>NN enhances sustained oscillations and homeostasis [115].</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X ↔ Y ↔ Z</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X ↔ Y ↔ Z</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.2 Network Motifs in Metal Ion Homeostasis and Signaling Systems

As mentioned in Section 1.3.3.2 and 1.3.3.3, metal ion homeostasis systems frequently contain transcriptional regulations on various proteins (ion channels or transporters, metal-using proteins, chaperones and regulatory proteins). Moreover, metal ion signaling systems are specific types of signal transduction systems. Therefore, it is not surprising that many network motifs found in the transcription networks and signal transduction networks also exist widely in the metal ion homeostasis and signaling systems. Here we will give a number of examples and then mainly focus on the signaling cycle motif and study the modulation on its properties by an inhibitor using mathematical model.

6.2.1 Examples of Network Motifs

Autoregulation motif is the simplest motif firstly defined in transcription networks. Positive autoregulation occurs when a transcription factor X activates the transcription of its own gene. In contrast, negative autoregulation occurs when X represses the
transcription of its own gene [10,11,193]. In metal ion homeostasis and signaling systems, many central transcriptional regulators have autoregulations. For instance, the activator Zap1 in yeast zinc homeostasis system (see Fig. 1.2) has positive autoregulation [133]. Fur, the critical repressor in the iron homeostasis system of *E. coli*, has weak negative autoregulation although Fur concentrations do not change significantly in response to iron availability [55]. The repressor CopY in *E. hirae* copper homeostasis system represses the transcription of its own gene [202]. However, no autorgulation of Zur, the critical repressor in the zinc homeostasis system in *E. coli*, was observed [167].

The second important family of network motifs is the feedforward loop. Because each of the three regulatory interactions (see Table 6.1, the regulations of X on Y, Y on Z and X on Z) in the FFL can be either activation or repression, there are totally eight possible structural types of FFL which can be classified into two groups (coherent and incoherent FFLs) as shown in Fig. 6.1 [10,11].

**Coherent FFL**

Coherent type 1

\[
\begin{align*}
X & \rightarrow Y \\
Y & \rightarrow Z
\end{align*}
\]

Coherent type 2

\[
\begin{align*}
X & \rightarrow Y \\
Y & \rightarrow Z
\end{align*}
\]

Coherent type 3

\[
\begin{align*}
X & \rightarrow Y \\
Y & \rightarrow Z
\end{align*}
\]

Coherent type 4

\[
\begin{align*}
X & \rightarrow Y \\
Y & \rightarrow Z
\end{align*}
\]

**Incoherent FFL**

Incoherent type 1

\[
\begin{align*}
X & \rightarrow Y \\
Y & \rightarrow Z
\end{align*}
\]

Incoherent type 2

\[
\begin{align*}
X & \rightarrow Y \\
Y & \rightarrow Z
\end{align*}
\]

Incoherent type 3

\[
\begin{align*}
X & \rightarrow Y \\
Y & \rightarrow Z
\end{align*}
\]

Incoherent type 4

\[
\begin{align*}
X & \rightarrow Y \\
Y & \rightarrow Z
\end{align*}
\]

**Figure 6.1. Coherent and incoherent FFLs.** In coherent FFLs, the indirect regulation of X on Z through Y is coherent with its direct regulation on Z. For example, in coherent type 1 FFL, X activates Y and Y activates Z, thus the indirect regulation of X on Z through Y is activation, which is coherent with the directly activating regulation of X on Z (this figure is taken from [11]).

FFL appears in hundreds of gene systems in various organisms. However, in metal ion homeostasis and signaling systems, FFL is not common. One example of FFL can be found in the zinc homeostasis system in *S. cerevisiae* (see Fig. 1.2), in which cytoplasmic zinc regulates the membrane concentration of Zrt1 both directly (excessive cytoplasmic zinc results in the inactivation of Zrt1 through endocytosis and vacuolar degradation) and indirectly (through Zap1) [133].
The third family of network motifs, SIM motif, exists extremely widely in metal ion and homeostasis and signaling systems because in these systems, it is very common that a single regulator has multiple targets. A good example of SIM motif found in yeast zinc homeostasis system has been described in Fig. 1.7d. Another SIM motif example is that Crz1, the central transcription factor in yeast calcium homeostasis/signaling system, regulates multiple target genes including \textit{PMC1} and \textit{PMR1} [208]. There are many other examples, for instance, in \textit{E. hirae} copper homeostasis system, CopY represses the expression of genes encoding CopA (the uptake ATPase), CopB (the efflux ATPase), CopZ (the chaperone) and itself [202].

Feedback loop motifs are very common in metal ion homeostasis systems. In addition to the example described in Fig. 1.7b, here we give more examples of feedback loop motifs as shown in Fig. 6.2.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{feedback_loop_motifs.png}
\caption{More examples of feedback loop motifs. (a). An example of feedback loop motif found in \textit{E. coli} iron homeostasis system. Cytoplasmic Fe\textsuperscript{2+} (i.e., \([\text{Fe}^{2+}]_i\)) in \textit{E. coli} represses the \textit{suf} operon (encoded by \textit{sufABCDSE}) which is involved in [Fe-S] cluster formation [191]. (b). A feedback loop example found in \textit{E. coli} iron homeostasis system. Cytoplasmic Fe\textsuperscript{2+} in \textit{E. coli} represses a small RNA (sRNA) named as RyhB, which facilitates the degradation of \textit{iscSUA} transcripts. \textit{iscSUA} are parts of the \textit{isc} operon (encoded by \textit{iscRSUA}) which are involved in [Fe-S] cluster formation [191]. (c). A feedback loop motif example found in the iron homeostasis system in \textit{S. cerevisiae}. In response to iron starvation, the iron-responsive transcription factors Aft1 and Aft2 activate Cth2 which facilitates the degradation of mRNAs encoding Fe-using proteins [142]. (d). A coupled negative feedback loop motif example found in the zinc homeostasis system in \textit{S. cerevisiae}. Under zinc-limiting conditions, zinc-responsive transcription factor Zap1 activates Zrt1. Moreover, excessive cytoplasmic zinc results in the inactivation of Zrt1 through endocytosis and vacuolar degradation [202].}
\end{figure}

Very frequently, biological cells use coupled feedback loop pathways for the regulations of metal ion and homeostasis and signaling systems. For example, the feedback loops shown in Fig. 1.7b and Fig. 6.2a-c are all coupled with each other and function together to maintain iron homeostasis in \textit{E. coli}. Fig. 6.2d shows a good example of coupled negative feedback loops motif (i.e., coupled NN motif; for the definition of NN, please
see Table 6.1) found in the yeast zinc homeostasis system. More examples of PN and NN network motifs are shown in Fig. 6.3.

**NN Examples**

(a) ZnuABC

(b) Pmr1

**PN Examples**

(c) ZRC1

(d) Zn(II) r-proteins

**Figure 6.3. Examples of coupled feedback loops (NN and PN) motifs.** (a). NN example found in E. coli zinc homeostasis system: the Zur-ZnuABC pathway is a negative feedback loop (NFL) and the ZntR-ZntA pathway is another NFL [163,172]. (b). NN example found in S. cerevisiae calcium homeostasis system: the CaM-Pmc1 pathway (more precisely, CaM-calcineurin-Crz1-Pmc1 pathway) is one NFL and the CaM-Pmr1 (more precisely, CaM-calcineurin-Crz1-Pmr1 pathway) pathway is another NFL [51,132]. (c). PN example found in S. cerevisiae zinc homeostasis system: the Zap1-ZRC1 pathway is a positive feedback loop (PFL) and the Zap1-ZRT1 pathway is a NFL [202]. (d) PN example found in Bacillus subtilis zinc homeostasis system: the Zur-Zn(II) r-proteins pathway is a PFL because Zur promotes mobilization of Zn(II) stored in Zn(II) r-proteins. In contrast, the Zur-yciABC pathway is a NFL [149]. CaM: calmodulin; yciABC: Zinc uptake transporter; Zn(II) r-proteins: Zn(II) containing ribosomal proteins.

**6.2.2 Signaling Cycle Motif**

Signaling cycle motif (see Table 6.1) is a cycle of covalent modification which is a ubiquitous building block of signaling pathways [83,84,213]. For example, in the Ca²⁺-calcineurin-MCIP-NFAT signaling network shown in Fig. 4.1, there are 8 enzyme catalytic binding reactions (see Reactions 10-17 in Table 4.1) which constitute 4 signaling cycle motifs (e.g., Reactions 10 and 11 forms a signaling cycle: MCIP[CaC⁰]MCIPp[CaC⁰]. Similarly, three other signaling cycles are formed by Reactions 12 and 13, Reactions 14 and 15, Reactions 16 and 17, respectively).
Although several modeling studies have been previously published about the properties of the signaling cycle motif [83,84], it is rarely noticed that in many cases, the appearance of signaling cycle is accompanied with an inhibitor as described in Fig. 6.4a. A good example (as described in Fig. 6.4b) is that protein 14-3-3 inhibits the NFAT signaling cycle formed by Reactions 14 and 15 in Table 4.1. Another example found in the signaling network shown in Fig. 4.1 is that protein 14-3-3 inhibits the MCIPPP signaling cycle formed by Reactions 12 and 13 in Table 4.1. What we are particularly interested in is how the accompanying inhibitor will modulate the properties of the signaling cycle motif.

**Figure 6.4. Signaling cycle with an inhibitor.** (a). In signaling cycle motif, Enzyme $E_1$ activates protein $W$ (to produce $W^*$ which is the active form of $W$) and $E_2$ deactivates it. The inhibitor $S$ binds with $W$ to form a complex $C$ which can not be activated by $E_1$. (b) An example of signaling cycle with an inhibitor found in Fig. 4.1. Here, protein 14-3-3 acts as the inhibitor.

The signaling cycle with an inhibitor can be represented as a set of three reactions in Cellerator form as follows:

$$
\left\{ \left[ W \rightarrow W^*, k_1, k_{-1}, k_2 \right\}, \left[ W^* \rightarrow W, k_3, k_{-3}, k_4 \right\}, \left[ S + W \rightarrow C, k_5, k_{-5} \right\}
$$

The ordinary differential equations used for simulating the signaling cycle with an inhibitor are generated automatically using Cellerator. The detailed 8 equations are:
dC(t) \over dt = -k_5 C(t) + k_3 S(t) W(t)
dE_1(t) \over dt = -k_1 E_1(t) W(t) + k_2 W \cup E_1(t) + k_3 W \cup E_1(t)
dE_2(t) \over dt = -k_2 E_2(t) W^* + k_3 W^* \cup E_2(t) + k_4 W^* \cup E_2(t)
dS(t) \over dt = k_2 C(t) - k_5 S(t) W(t)
dW(t) \over dt = k_5 C(t) - k_3 S(t) W(t) - k_1 E_1(t) W(t) + k_3 W^* \cup E_2(t) + k_4 W \cup E_1(t)
dW^*(t) \over dt = -k_3 E_2(t) W^*(t) + k_4 W^* \cup E_2(t) + k_2 W \cup E_1(t)
dW^* \cup E_2(t) \over dt = k_1 E_1(t) W^*(t) - k_4 W^* \cup E_2(t) - k_2 W \cup E_1(t)
dW \cup E_1(t) \over dt = k_4 E_1(t) W(t) - k_2 W \cup E_1(t) - k_1 W \cup E_1(t)

Where C(t), S(t), W(t), W^*(t), E_1(t) and E_2(t) denote the concentrations of species C, S, W, W*, E_1 and E_2 respectively. W \cup E_1(t) denotes the concentration of the intermediate complex formed by W and E_1. W^* \cup E_2(t) denotes the concentration of the intermediate complex formed by W* and E_2. k_1, k_2, k_3, k_4, k_5, k_{-2} are rate parameters.

We numerically solve the above equations for various values of the total concentrations of the inhibitor (i.e., S^{tot}) and depict the steady state concentration of W^* (i.e., W^{ss}) as a function of the total concentration of E_1 (i.e., E_1^{tot}) as shown in Fig. 6.5a.

Figure 6.5. Modulation on the steady-state response of the signaling cycle by an inhibitor. (a) The black, blue, green and red curves describe the steady-state response curves
(\(W^{\text{ss}}\) as a function of \(E_1^{\text{tot}}\)) for \(S^{\text{tot}} = 0, 400, 1000, 2000\), respectively. The rest parameter values are as follows: \(k_1 = k_4 = k_3 = k_9 = k_5 = k_7 = 1, E_2^{\text{tot}} = 10, W^{\text{tot}} = 2000\). \(W^{\text{tot}}\) denotes the total concentration of \(W\). In the simulations, the following initial conditions are used:

\[E_1(0) = E_2^{\text{tot}}, E_2(0) = E_2^{\text{tot}}, S(0) = S^{\text{tot}}, W(0) = W^{\text{tot}}\]

and the initial values of all the rest variables are 0. (b) The black and green solid curves describe the steady-state concentration of \(W\) (i.e., \(W^{\text{ss}}\)) as a function of \(E_1^{\text{tot}}\) for \(S^{\text{tot}} = 0\) and \(S^{\text{tot}} = 1000\), respectively. The dashed green line describes the steady-state concentration of \(C\) (i.e., \(C^{\text{ss}}\)) as a function of \(E_1^{\text{tot}}\) for \(S^{\text{tot}} = 1000\).

From Fig. 6.5a, we can see that for various values of \(S^{\text{tot}}\), \(W^{\text{ss}}\) hardly increases (from 0) when \(E_1^{\text{tot}} < 9\). For \(S^{\text{tot}} = 0\), \(W^{\text{ss}}\) rises to less than 200 when \(E_1^{\text{tot}}\) increases from 9 to 9.9, then increases extreme rapidly to more than 1800 when \(E_1^{\text{tot}}\) increases from 9.9 to 10.1 and finally rises very gradually in a hyperbolic way to approximate its limit value (i.e., 2000) when \(E_1^{\text{tot}}\) increases from 10.1 to 20. We name the narrow range of \(E_1^{\text{tot}} (9.9 \leq E_1^{\text{tot}} \leq 10.1)\) as the critical region. For \(S^{\text{tot}} = 400\), the end value of \(W^{\text{ss}}\) during this critical region is around 1500, which is lower than that for \(S^{\text{tot}} = 0\). For \(S^{\text{tot}} = 1000\), the end value of \(W^{\text{ss}}\) during this critical region is even lower (less than 1000) whereas for \(S^{\text{tot}} = 2000\), there is little change of \(W^{\text{ss}}\) during this critical region.

From Fig. 6.5b, we can see that for the signaling cycle without inhibitor (i.e., \(S^{\text{tot}} = 0\), the black curve), the steady-state profile of \(W^{\text{ss}}\) is almost mirror symmetrical to that of \(W^{\text{ss}}\) (i.e., the black curve in Fig. 6.5a) along the horizontal line \(W^{\text{ss}} = 1000\). This is because in this case, \(W(t) + W^*(t) \approx W^{\text{tot}} = 2000\) (Please note that

\[W(t) + W^*(t) + W \cup E_1(t) + W^* \cup E_2(t) = W^{\text{tot}} = 2000\]

and

\[W \cup E_1(t) + W^* \cup E_2(t) \leq E_1^{\text{tot}} + E_2^{\text{tot}} \leq 30\). For the signaling cycle with an inhibitor (i.e., \(S^{\text{tot}} = 1000\), the green curve in Fig. 6.5b), when \(E_1^{\text{tot}} < 9\), both \(W^{\text{ss}}\) and \(C^{\text{ss}}\) are around 1000. Then \(W^{\text{ss}}\) extreme rapidly decreases to almost 0 during the critical region whereas \(C^{\text{ss}}\) gradually decreases when \(E_1^{\text{tot}}\) increases from 9.9 to 20 and the decreasing rate of \(C^{\text{ss}}\) is almost equal to the increasing rate of \(W^{\text{ss}}\) shown in Fig. 6.5a (the green curve) because in this case, \(W(t) + W^*(t) + C(t) \approx W^{\text{tot}} = 2000\).

6.3 Discussion

As we can see from Section 6.2.1, several kinds of network motifs such as autoregulation motif, SIM motif, feedback loop motif and coupled feedback loop motifs do exist widely in metal ion homeostasis and signaling systems. It is interesting to notice that relatively simple network motifs can be embedded in more complex network motifs, for instance, the coupled NN motif shown in Fig. 6.2d contains a FFL motif ([Zn\(^{2+}\)], regulates Zrt1 both directly and indirectly). The coupled NN motif shown in Fig. 6.3a contains a SIM motif ([Zn\(^{2+}\)], regulates both Zur and ZntR).
The great number of negative feedback loops shown in Fig. 6.2b-d and Fig. 6.3 confirms our previous statement that negative feedback controls constitute the central scheme for maintaining homeostasis. The various strategies used by biological cells for maintaining homeostasis as discussed in Section 1.3.3.2 and 1.3.3.3 can be represented in the framework of network motifs (especially feedback loop motifs). Thus investigations of the properties of the network motifs are quite important in order to gain insight into the general designing principle of metal ion homeostasis and signaling systems.

The most well-known characteristic of signaling cycle is that the steady-state response of this basic cycle can be in a highly sigmoidal (ultrasensitive) regime as can be clear seen in the behavior of the black curve in Fig. 6.5a during the critical region [83,84]. Inhibitor S can modulate the steady-state response of the signaling cycle by suppressing the amplitude of the value change of $W^{*\text{ss}}$ during the critical region and increasing the rate of the value change of $W^{*\text{ss}}$ after the critical region (see Fig. 6.5a). The investigations shown here can help us understand why signaling cycle can function as the pivot where the interaction of multiple signaling pathways happens (e.g., the interaction between 14-3-3-NFAT pathway and calcineurin-NFAT pathway takes place through the signaling cycle shown in Fig. 6.4b. The interaction between the BMK1/ERK526 signaling pathway and calcineurin-MCIP pathway happens through the MCIP$^p$ signaling cycle formed by Reactions 10 and 11 in Table 4.1). It is known that three simple systems (activator-inhibitor system, substrate-deplete system and delayed negative feedback system) containing a signaling cycle can generate oscillating behavior [213]. We will further investigate the modulating effect on the oscillating behavior of these systems after adding an inhibitor to the signaling cycle in these systems.

In this Chapter, we gave a concise summary of the functions of various network motifs in cellular networks and enumerated those found in metal ion homeostasis and signaling systems. Particularly, we used simulation results of an ODE model to show the modulation effect on the properties of the signaling cycle motif by an inhibitor. In Chapter 7, we will summarize the thesis, do some final discussions and indicate the future work.

---

26 ERK5 is a synonym of BMK1 [95].