

## Crucial function of histone lysine methylation in plant reproduction

YAO XiaoZhen<sup>1\*</sup> & SHEN WenHui<sup>2\*</sup>

<sup>1</sup> State Key Laboratory of Genetic Engineering, Department of Biochemistry, School of Life Sciences, Fudan University, Shanghai 200433, China;

<sup>2</sup> Institut de Biologie Moléculaire des Plantes du CNRS, Université de Strasbourg, 12 rue du Général ZIMMER, 67084 STRASBOURG, France

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Unlike animals, plants do not set aside germ cells early in development. In angiosperm species, reproduction occurs in the adult plant upon flowering. The multicellular male and female gametophytes differentiate from meiotic products within reproductive floral organs. Double fertilization is another remarkable feature of most angiosperm species. The zygote derived from fertilization of the egg cell by one of the sperm cells and the endosperm from fertilization of the central cell by the second sperm cell develop in a coordinated manner together and enclosed in the sporophytic maternal integuments, forming the seed. Understanding plant reproduction is biologically pertinent and agronomically and ecologically important. Here, we describe the known functions of histone lysine methylations in various steps of reproduction in the reference plant *Arabidopsis thaliana*. It is emerging that histone lysine methylation is key for understanding epigenetic regulation networks of genome function.

**epigenetics, chromatin, histone methylation, SET domain, methyltransferase, *Arabidopsis thaliana***

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In eukaryotes, DNA is packaged into a structure called chromatin. The fundamental unit of chromatin is nucleosome, which is composed of 146 base pairs of DNA wrapped around a histone octamer (two copies of each histone proteins H2A, H2B, H3, and H4). The structure properties of chromatin determine the DNA accessibility to various types of protein factors and RNA molecules and thus greatly influence the genome function. Epigenetics refer to heritable (mitotically and/or meiotically) changes in genome function that occur without a change in DNA sequence. Well-known epigenetic mechanisms include posttranslational covalent modifications (e.g. methylation and ubiquitylation) of histone tails, histone variant replacement, ATP-dependent chromatin remodelling and DNA methylation at cytosine residues.

Methylation of lysine residues on the tails of histones plays key roles in the regulation of gene expression and chromatin structure in eukaryotes. In *Arabidopsis*, histone lysine methylation occurs mainly at histone H3 lysines 4, 9,

27 and 36 (H3K4, H3K9, H3K27 and H3K36) [1]. In general, H3K4 and H3K36 methylation correlates with transcriptional activation whereas H3K9 and H3K27 methylation associates with gene silencing. In addition, each lysine can be mono-, di- or tri-methylated, and these different methylation states could have distinct physiological effects [2]. Histone methylations are carried out by histone methyltransferases that contain an evolutionarily conserved domain, the SET (named after SU(VAR) 3–9, E(Z) and TRITHORAX in *Drosophila*) domain. The reference plant *Arabidopsis thaliana* contains over 30 genes encoding SET-domain proteins [3], and so far only some of these genes have been functionally characterized [4,5].

The plant life cycle alternates between diploid sporophytic generation and a much reduced haploid gametophytic generation. In most of sexually reproducing plants including *Arabidopsis*, the gametophytic generation occurs late in development within sporophytic tissues of specialized floral organs. After pollination, the male and female gametes fuse

\*Corresponding authors (email: xiaozhenyao@163.com; wen-hui.shen@ibmp-cnrs.unistra.fr)

to produce diploid zygote and triploid endosperm. Histone methylations play pivotal roles throughout the plant life cycle. Here, we discuss crucial functions of histone lysine methylations in *Arabidopsis* reproduction, including flowering transition, gametophyte development, fertilization and seeds development.

## 1 Histone lysine methylations in flowering transition

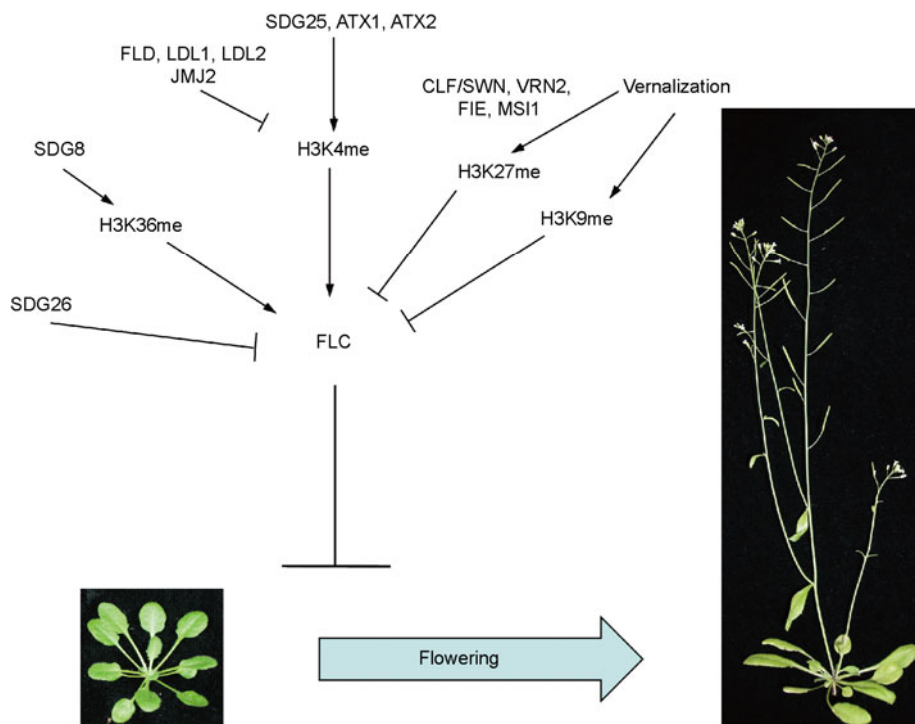
Flowering, which represents the transition from the vegetative to reproductive development, is critical to reproductive success. The *Arabidopsis* flowering transition is controlled by four major regulatory pathways, namely photoperiod, vernalization, gibberellic acid signaling, and autonomous pathways [6]. FLOWERING LOCUS C (FLC), a MADS box transcription factor, is a major repressor of flowering and plays a central role in flowering-time control [7,8]. Several SET DOMAIN GROUP (SDG) proteins are involved in deposition of either 'active' or 'repressive' methylation marks at *FLC* chromatin, and a fine-tuned chromatin remodeling at *FLC* is crucial for flowering time control (Figure 1).

### 1.1 H3K36 and H3K4 methylations in activation of *FLC* expression

*SDG8/ASHH2*, also known as *Early Flowering in Short*

*days (EFS)*, is the first *SDG* gene shown to be involved in activation of *FLC* expression [9,10]. The *sdg8* mutants display an early-flowering phenotype, which is associated with reduced levels of *FLC* expression [9–11]. Ectopic expression of *FLC* is sufficient to inhibit the early-flowering phenotype in *sdg8* mutant background [10]. *Arabidopsis* has 5 FLC paralogs, namely MADS AFFECTING FLOWERING1 (MAF1) to MAF5. While *MAF2* and *MAF3* expression is barely affected, expression of *MAF1*, *MAF4* and *MAF5* is strongly reduced in the *sdg8* mutants [11]. Nevertheless, whether reduced *MAF* gene expression contributes to the *sdg8* mutant phenotype remains unexamined. The SDG8 protein shows sequence homologies with the yeast H3K36-methyltransferase SET1/SET2. Consistently, a reduced level of H3K36me2 was observed in global histone extract as well as at the *FLC* and *MAF* loci in the *sdg8* mutants [10,11]. Interestingly, while the level of H3K36me3 was also reduced, H3K36me1 accumulated to higher levels in the *sdg8* mutants, indicating that SDG8 is responsible for deposition of H3K36me2 and H3K36me3 but not H3K36me1 [11]. This also establishes that the levels of H3K36me2 and H3K36me3 but not H3K36me1 positively correlate with transcriptional activation of *FLC* and *MAF* genes. It will be obviously interesting to understand how the H3K36 methylation states are controlled and how the SDG8 protein is targeted to *FLC* and *MAF* loci.

The SDG26/ASHH1 protein is smaller and shares sequence homologies with only the C-terminal part of SDG8/ASHH2, including the SET domain. Strikingly, the *sdg26*



**Figure 1** Histone methylations fine-tune *FLC* expression in the control of transition to flowering. Solid lines with arrows indicate up-regulation or activation of gene expression, and solid lines with bars for down-regulation or repression of gene expression.

mutants, opposite to the *sdg8* mutants, show a late-flowering phenotype and up-regulation of *FLC*, *MAF4* and *MAF5* [11]. Although the recombinant SDG26 protein could methylate oligonucleosomes *in vitro*, histone methylation levels were barely affected in the *sdg26* mutant plants. Molecular mechanisms involved in the SDG26-mediated repression of *FLC* and *MAF* genes remain obscure so far.

In addition to SDG8 and SDG26, three members of the Trithorax (TRX) group in *Arabidopsis*, ATX1/SDG27, ATX2/SDG30 and ARABIDOPSIS TRITHORAX-RELATED7 (ATXR7)/SDG25, also contribute to flowering transition control. The *atx1* mutant shows a weak early-flowering phenotype, which is enhanced by additional knockdown of ATX2 [12]. The ATX1 protein directly interacts with *FLC* chromatin and is involved in the H3K4me3 deposition at the *FLC* locus [12]. FRIGIDA (FRI), a coiled-coil protein, acts to activate *FLC* expression and inhibits flowering [13]. It has been reported that WDR5a, a homology of human WDR5 (one of the 4 core components of the MLL1 complex, which catalyzes H3K4 methylation), interacts with ATX1 in the yeast two-hybrid assay, and is required for FRI-mediated H3K4me3 deposition at *FLC* chromatin [14]. The FRI-based complex also contains SDG8/EFS involved in H3K36me3 deposition at *FLC* chromatin [15,16]. Although ATX1 and ATX2 share high sequence homologies, ATX1 is involved in H3K4me3 whereas ATX2 in H3K4me2 deposition [17]. Nevertheless, whether ATX2 and ATX1 act sequentially on H3K4me2 and H3K4me3 deposition at *FLC* remains unexamined so far.

Compared to ATX1 and ATX2, SDG25/ATXR7 plays a more substantial role in flowering repression. The *sdg25/atxr7* mutants show an obvious early-flower phenotype and reduced levels of *FLC* expression [18,19]. The recombinant SDG25/ATXR7 protein could methylate oligonucleosomes *in vitro* [18] and the SDG25/ATXR7-GFP protein was located around the transcript start site of *FLC* [19]. Modest reduction of H3K4me2, H3K4me3 and H3K36me2 levels was observed at *FLC* in the *sdg25/atxr7* mutant plants [18,19]. The *atx1 sdg25/atxr7* double mutant showed an enhanced early-flowering phenotype and more drastic reduction of H3K4me2 and H3K4me3 at *FLC* [19]. Molecular interaction between ATX1 and SDG25/ATXR7 remains unclear.

## 1.2 H3K27 methylation in repression of *FLC* expression

Polycomb Group (PcG) proteins were initially characterized in *Drosophila melanogaster* through their crucial function in silencing of homeotic (*Hox*) genes [20]. They exist in multi-protein complexes; the best characterized are known as polycomb repressive complex1 (PRC1) and PRC2. PRC2 is responsible for H3K27me3 deposition and is composed of 4 core proteins, e.g. Enhancer of zeste (E(z), an H3K27-

methyltransferase), Suppressor of zeste 12 (Su(z)12), Extra sex comb (Esc) and p55 [20]. H3K27me3 is recognized by the core component Pc of the PRC1 complex, and PRC1 further modifies chromatin by H2A monoubiquitylation [20]. The composition of PRC2 is conserved in *Arabidopsis* [21]. Homologs of all 4 core members of PRC2 have been identified in *Arabidopsis*: CURLY LEAF (CLF/SDG27), MEDEA (MEA/SDG1), and SWINGER (SWN/SDG30) are E(Z) homologs; FERTILIZATION INDEPENDENT SEED2 (FIS2), EMBRYONIC FLOWER2 (EMF2), and VERNALIZATION2 (VRN2) are Su(z)12 homologs; MULTICOPY SUPPRESSOR OF IRA (MSI) 1–5 are p55 homologs; and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) is the only one homolog of Esc [21]. More recently, PRC1-like complexes were also characterized in *Arabidopsis* and shown to play essential roles in plant development [22–24].

Prolonged exposure in cold (vernalization) during winter is memorized for blooming in spring in many plant species, including some agronomically important cereals. In *Arabidopsis*, vernalization promotes flowering largely by repression of *FLC* expression [25]. Vernalization increases H3K9me2, H3K27me2 and H3K27me3 accumulation at *FLC* locus [26]. PcG proteins VRN2, FIE, CLF/SDG27, SWN/SDG30, MSI1 and the plant homeodomain proteins VERNALIZATION INSENSITIVE 3 (VIN3) and VIN3-like1 (VIL1/VRN5) form large protein complexes involved in the H3K27me3 deposition on *FLC* chromatin during vernalization [27]. H3K27me3 spreads across the *FLC* locus in a DNA replication dependent manner [28]. The PRC1-like component, TERMINAL FLOWER2 (TFL2)/LIKE HETEROCHROMATIN PROTEIN1 (LHP1), associates with chromatin regions enriched of H3K27me3 and is involved in the maintenance of *FLC* repression [29]. The flowering phenotype caused by knockdown of PcG genes is complex. Opposite to a late-flowering phenotype predictable by release of *FLC* repression, the *clf*, *emf2* and *lhp1* mutants display a strong early-flowering phenotype. This is now understood because that PRC2 and LHP1 play key roles in repression of *FLOWERING LOCUS T* (*FT*), a major flowering activator acting at downstream of *FLC* in the regulatory pathway [30].

## 1.3 Fine-tuning histone lysine methylations in regulation of *FLC* expression

Distinct from single mutants, the *atx1 clf* double mutant showed a phenotype indistinguishable from wide-type plants [31]. It was shown that ATX1 and CLF proteins interact physically with each other in the yeast two-hybrid assay [31]. This study unravels antagonistic roles of ATX1 and CLF. Nevertheless, much progress needs to be made to understand how different histone methylations mark the *FLC* chromatin spatially and timely in the flowering time control.

Histone methylations written by SDG histone methyl-

transferases can be erased by histone demethylases. FCA, an RNA-binding protein, plays roles in the autonomous pathway for flowering time control. FLOWERING LOCUS D (FLD), a plant homolog of Lysine-Specific Demethylase1 (LSD1, a H3K4-demethylase), is required for FCA to down-regulate *FLC* expression and promote flowering [32]. The other two homologues of LSD1, LSD1-LIKE1 (LDL1) and LSD1-LIKE2 (LDL2), also play partially redundant roles with FLD in the regulation of floral transition [33]. JmjC-domain proteins are known to have histone demethylase activity. So far, three proteins that belong to Jumonji class have been shown to control the flowering transition. RELATIVE OF EARLY FLOWERING6 (REF6)/AtJmj2 acts as an *FLC* repressor, and the *ref6* mutant shows late flowering phenotype under both long-day and short-day growth conditions [34]. AtJmj4/14 and EARLY FLOWERING6 (ELF6)/AtJmj1, acting as H3K4 demethylases, repress *FT* expression and prevent precocious flowering in *Arabidopsis* [35,36]. It was reported that ELF6/AtJmj1 and AtJmj4/14 bind *FT* chromatin and that H3K4 methylations are increased in the *elf6* and *jmj4* and more pronouncedly in the double *elf6 jmj4* mutants [35]. Increase of H3K4me2/me3 levels was also observed at *FLC* chromatin in the *ref6* and *fld* mutants [15].

Small interfering RNA (siRNA) could regulate transcription via histone methylation. In the *Arabidopsis* Ler accession, *FLC* contains a transposable element inserted in an intron of the gene, and siRNAs were shown to be involved in targeting *FLC* for repressive H3K9 methylation [37]. In the *Arabidopsis* Columbia (Col) accession, two small RNAs corresponding to the reverse strand were identified and shown to be involved in H3K9 methylation at *FLC* in a *DICER-LIKE2* (*DCL2*)/*DCL3*/*DCL4*-dependent manner [38]. Interestingly, mutations for a single *DCL* do not trigger a strong flowering phenotype, while double *dcl1 dcl3* mutants are late flowering due to an increase of *FLC* expression [39]. The identity of SDG(s) involved in H3K9 methylation at *FLC* locus remains currently unknown. Recently, a long intronic noncoding RNA, named COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR), was shown to be required for the vernalization-mediated epigenetic repression of *FLC* [40]. COLDAIR physically associates with CLF, suggesting that COLDAIR establishes stable repressive chromatin at *FLC* through recruiting PRC2 in H3K27me3 deposition [40].

## 2 Function of histone lysine methylations in gametophyte development

Female gametophytes or megagametophytes develop in ovules within the gynoecium of the *Arabidopsis* flower. A single megaspore mother cell (megasporocyte) differentiates from the subepidermal cell layer at the tip of each ovule primordium and undergoes meiosis to produce a tetrad of

four haploid spores. Three of the spores degenerate, and one proceeds through 3 sequential rounds of mitotic division, forming the female gametophyte, the embryo sac, which at maturation consists of 7 cells with four cell types (3 antipodal cells, 2 synergid cells, 1 egg cell and 1 two-haploid-fused diploid central cell). The male gametophytes or microgametophytes develop within anthers of the *Arabidopsis* flower. Microsporocytes differentiate from the primary sporogenous tissue surrounded by the tapetum and undergo meiosis to form a tetrad of four haploid microspores. The nucleus of each microspore follows one cycle of nuclear division, forming a generative cell and a vegetative cell. The generative cell undergoes one more round of mitosis to produce two sperm cells. At maturation the male gametophyte, the pollen grain, is thus composed of a three-celled male germ unit.

Besides controlling the flowering time, SDG8/ASHH2 is also involved in floral organ as well as male and female gametophyte development [11,41]. It was reported that about 80% of *sdg8/ashh2* mutant mature ovules lack an embryo sac [41]. As to the male gametophyte development, the tapetum development was affected and the number of functional pollen per anther is drastically reduced in *sdg8/ashh2*. Furthermore, the transmission efficiency of *sdg8/ashh2* was reduced through both male and female gametes. Reduced levels of H3K36me3 were observed at the floral organ identity regulatory gene *API*, the meiosis essential gene *AtDMC1* and the pollen development regulatory gene *MYB99*, suggesting that SDG8/ASHH2-mediated H3K36me3 deposition is important for activation of these genes, which is required during various processes of reproduction [41].

More recent characterization of the *SDG2/ATXR3* gene revealed that H3K4 methylation is crucial for plant reproduction [42,43]. Compared to the partial sterile phenotype of the *sdg8/ashh2* mutants, the *sdg2* mutants are completely sterile. SDG2 is a methyltransferase responsible for the global genome-wide H3K4me3 deposition. It is involved in multiple developmental processes, remarkably important in initiation processes of female and male gametophyte development [42]. Although both *sdg2* and *sdg8/ashh2* mutants show floral organ and gametophyte defects, they also display obvious differences. Future analysis by the use of developmental marker genes might be helpful to precise SDG2 and SDG8/ASHH2 roles during gametogenesis. Transcriptome analyses of young flower buds have identified a few hundred genes differentially regulated by more than 2-fold in either *sdg2-1* or *sdg8/ashh2* mutant [41,42]. Only less than 20% of differentially expressed genes are in common between the two mutants and these are primarily involved in metabolism and transport, indicating that SDG2 and SDG8/ASHH2 may regulate flower development through common and distinct gene networks. Among the SDG2-target genes, the MADS-like transcription factor gene *SPOROCTELESS/NOZZLE* (*SPL/NZZ*) is of particular interests [42]. This gene acts upstream of many stamen-expressed and ovule-

expressed genes and is required for initiation of both microsporogenesis and megasporogenesis [44,45]. The only previous known gene that acts upstream of *SPL/NZZ* is the reproductive organ identity gene *AGAMOUS* (*AG*), which activates *SPL/NZZ* expression through binding to the 3'-region of the *SPL/NZZ* gene [46]. The *SPL/NZZ* locus contains bivalent chromatin domains consisting of the transcriptional active mark H3K4me3 and the transcriptional repressive mark H3K27me3 [42]. SDG2 is required for marking H3K4me3 for active transcription of *SPL/NZZ*. However, PcG genes involved in marking H3K27me3 at *SPL/NZZ* remains currently unknown. Study of SDG2-mediated activation and PcG-mediated repression of *SPL/NZZ* might provide a key step for understanding gene regulatory networks in gametophyte development.

### 3 Function of histone lysine methylations in seed development

Double fertilization is a specific event during plant reproduction. After pollination, the pollen tube delivers two sperm cells into embryo sac. One of the sperm cell fuses with egg cell, leading to development of a diploid embryo. Fusion of the other sperm cell with central cell generates the triploid endosperm, a nutritive tissue that supports embryogenesis. It has been reported that histone methylations play important roles in fertilization and seeds development.

Mutants *fie*, *fis2* and *mea/sdg1* show a similar phenotype that is autonomous endosperm development without fertilization [47–49]. The *mea* embryos display excess cell proliferation and enlarge radially symmetrically from later globular stage. *MEA* also controls the endosperm development through suppression of proliferation of the central cell before fertilization. When fertilization is prevented by remove the anther, the *mea* endosperm proliferates without embryo development [47,49]. The *mea* seeds turn white and collapsed during seed desiccation, and such kind of abnormality is independent of the paternal contribution and gene dosage. *MEA* is one of the best-characterized imprinted genes in plants. The maternal allele of *MEA* is hypomethylated and is actively expressed in the endosperm. A PRC2 complex, composed of FIE, FIS2, MSI1 and maternal-expressed MEA, is proposed to silence through H3K27-methylation the paternal *MEA* allele in endosperm cells [50]. This same complex is also likely responsible for suppression of central cell proliferation before fertilization. Similar to *mea*, *fie* and *msi1* shows endosperm proliferation independent from fertilization. In *msi1* mutant, the development of the embryos surrounded by diploid endosperm will arrested at different stages after pollination [51]. The *swn/sdg30* mutant alone does not show obvious defects; it can, however, enhance the *mea* mutant in producing fertilization independent seeds [52], indicating that additional PRC2 complexes may be involved in seed development control.

One of the targets of the MEA-based PRC2 complex is the MADS-box gene *PHERES1* (*PHE1*) [53]. In contrast to maternally expressed *MEA*, *PHE1* is preferentially expressed in endosperm from the paternal allele. The vegetative and reproductive development of *phe1* mutants was indistinguishable from that of wild-type plants. Interestingly, the repression of the maternal *PHE1* allele is disrupted in *mea* mutant seeds with mutated maternal *MEA* allele [54]. *MEA* and *FIE* directly bind with the *PHE1* promoter and repress the maternal *PHE1* allele through H3K27me3 deposition [53,54].

By monitoring *in situ* in the ovule the dynamics of chromatin states in the female gametes, Pillot et al. [55] showed that H3K9me2 and LHP1 are more abundantly present in the egg cell than in the central cell, which pre-establishes the differentiation that the embryo is relatively quiescent, while the endosperm is transcriptionally active. KRYPTONITE (KYP)/SDG33 is the methyltransferase primarily involved in H3K9me2 deposition in the embryo [55]. At 8 d after pollination, the entire endosperm genome is demethylated, which is in sharp contrast with the methylated embryo genome [56,57]. It thus appears that H3K9me2 associates with DNA methylation, contributing to the transcriptionally repressive heterochromatic state in the embryo, whereas hypomethylation of DNA and H3K9 are virtually linked to the permissive euchromatic state in the endosperm.

### 4 Concluding remarks

Studies over the last few years have shown that SDGs-mediated histone methylations play crucial roles in plant reproduction. The key flowering time regulator *FLC* is emerging as one of the most studied loci in epigenetic research. Because of its bivalent chromatin nature and its critical function in initiation of both microsporogenesis and megasporogenesis, *SPL/NZZ* may become a target of choice in future research. It is predictable that more factors will be identified as being involved in writing, reading and erasing histone methylations in the regulation of *FLC* and *SPL/NZZ* transcription. Still very little is known about how different factors involved in histone methylations are recruited to their chromatin targets. The interactions between different factors in many cases remain elusive or in some are totally unknown. We also need to dissect dynamics of histone methylations in temporal and tissue or cell-type specific manner. It will be challenging to understand how a histone methylation state is established and inherited during mitosis and/or meiosis, and how resetting of chromatin state occurs during reproduction.

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