Supporting Online Material for

*Arabidopsis* Type I Metacaspases Control Cell Death

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MATERIALS AND METHODS

Plant Materials and Growth Conditions
We used Arabidopsis thaliana Columbia (Col-0) and isogenic T-DNA insertional lines for lsd1 (At4g20380; SALK_042687 (S1); insertion at nucleotide 57 of intron four; Arabidopsis Biological Resource Center at Ohio State University (ABRC)), atmc1 (At1g02170, GABI_096A10; insertion at nucleotide 312 of exon one; GABI-Kat Resource, Bielefeld University, Germany) and atmc2 (At4g25110; SALK_009045; insertion at nucleotide 255 of exon one; ABRC). All insertion sites were re-confirmed by DNA sequencing. Plants were grown under short day conditions (9 hrs light, 21°C; 15 hrs dark, 18°C).

DNA Constructs
For conditional expression of AtMC1 and AtMC2 and their respective mutant alleles, we cloned the appropriate full-length or truncated, C-terminally HA-epitope-tagged cDNAs into a modified version (BASTAR) of vector pTA7002 (S2). A pBAR vector containing LSD1 cDNA C-terminally tagged with 6-myc and under the control of its native promoter as described in (S3) was used in co-immunoprecipitation experiments. For expression of AtMC1 and AtMC2 under control of their native promoters, HA-tagged AtMC1 and AtMC2 cDNAs were fused by PCR to their respective native promoters, directionally cloned into a pENTR/D/TOPO Gateway vector (Invitrogen) and recombined into the plant binary Gateway-compatible vector pGWB1 (S4).

For co-immunoprecipitation assays, AtMC1 and AtMC2 cDNAs were directionally cloned into a pENTR/D/TOPO Gateway vector (Invitrogen) and recombined into the plant binary Gateway-compatible vector pMDC7 (S5) with an Estradiol-inducible promoter and C-terminally-tagged with six myc epitopes (Erica Washington and Tim Eitas, personal communication).

DNA constructs were transformed by electroporation into Agrobacterium tumefaciens strains C58C1 (for transient transformation of N. benthamiana leaves) or GV3101 (for stable transformation of Arabidopsis and transient transformation of N. benthamiana leaves).

To inhibit post-transcriptional gene silencing in N. benthamiana leaves transformed with A. tumefaciens GV3101, the tomato bushy stunt virus silencing suppressor p19 under a 35S promoter (S6) was included in the infiltration mixture when pTA7002 or pBAR vectors were used.

Agrobacterium-Mediated Transformation
Arabidopsis transgenics were generated using A. tumefaciens (GV3101)-mediated transformation following the floral dip method as previously described (S7). Homozygous transgenic lines were selected on 50 µg/ml Hygromycin MS plates (pGWB1 and pMDC7) or by Basta (glufosinate-ammonium) selection (pTA7002 BASTA(S)).

For N. benthamiana transient leaf transformation assays, A. tumefaciens C58C1 (pMDC7) or GV3101 (pTA7002 and pBAR) was grown overnight at 28°C on 5 ml of KB and antibiotics. Pelleted cells were resuspended in infiltration media (10 mM MES, pH 5.6, 10 mM MgCl₂, and
150 µM acetosyringone) and incubated at room temperature with constant rotation. After 90 minutes, cells were resuspended to the appropriate OD600. Five to six-week-old N. benthamiana plants were inoculated by infiltration using a syringe. Infiltreated plants were kept under low light to allow transgene expression and 72 hours post-inoculation (hpi) samples were collected for further processing. Estradiol (Est) or Dexamethasone (Dex) were applied 48 h (hpi) when needed for induction of expression.

**Chemical Treatments**
Est or Dex (2 µM) supplemented with 0.005% Silwet were applied to N. benthamiana leaf surfaces using cottonballs.

For conditional induction of AtMC1 in stable Arabidopsis transgenics, plants were sprayed once with 20 µM Dex supplemented with 0.005% Silwet. For conditional expression of AtMC2, 6-day-old plants were sprayed with 1 µM Dex. Subsequently, plants were sprayed every five days with 1 µM Dex until harvested for experiments.

For induction of lsd1 runaway cell death, two- to four-week-old plants were sprayed with 150 µM benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH (0.005% Silwet)).

To monitor oxidative stress, fully expanded leaves of 4-week-old plants were infiltrated with a fresh mixture of 100 µM xanthine and 0.05 U ml⁻¹ xanthine oxidase (X/XO).

**Stains**
Dead cells and fungal structures were visualized with Trypan Blue as described (S8, S9).

For Magenta-Gluc (5-bromo-6-chloro-3-indolyl-β-D-glucuronide; Applichem) stains, leaves were vacuum infiltrated with 1 mM Magenta-Gluc solution, and incubated over night at 37°C. Subsequently, leaves were cleared with 70% ethanol. For Magenta-Gluc/Aniline Blue double staining, ethanol-cleared, stained leaves were additionally incubated overnight in a 0.5% Aniline Blue (in 0.15 M K₂HPO₄) solution (S10, S11).

For Magenta-Gluc/Trypan Blue double staining, Magenta-Gluc stained leaves were incubated in boiling TB for 5 min, and then cleared with chloral hydrate.

In order to visualize accumulation of H₂O₂, leaves from 4-week-old plants grown under short-day conditions were stained with 3,3'-Diaminobenzidine (DAB) 3 days after infiltration with X/XO as previously described (S12).

**Cell Death Assays**
We tested the mutants presented in Figure 4B with concentrated inoculum (5 x 10⁷ cfu/ml), and did not detect any differences in macroscopic HR or ion leakage. Thus, we employed lower inoculums to uncover the dose-sensitive HR phenotypes presented. We believe that this approach is justified, as it more closely resembles the low level inocula of bacterial infection occurring in nature. Our interpretation is that at extremely high doses of bacteria that we and others typically use for HR induction, several independent cell death systems are induced.
subsequent to NB-LRR-mediated pathogen recognition. By lowering the dose, we not only more closely mirror natural infections, but we also resolve local infections, in the same way that use of a sporulating oomycete like *Hyaloperonospora arabidopsidis* (*Hpa*) allows analysis of single cell infections sites.

Low inoculum, single cell death assays were adapted from Hatsugai *et al.* (S13): leaves were vacuum infiltrated with either 250,000 cfu/ml (Fig 4B) or 500,000 cfu/ml (Fig. 4C and fig. S6E) *Pseudomonas syringae pv tomato* DC3000(*avrRpm1*) and incubated for 12 hours at RT. Leaves were then harvested and stained with Trypan Blue (S8) to visualize dead cells.

Conductivity measurements: three- or four-week-old-plants treated with the appropriate inducer of cell death (see Chemical Treatments) were harvested. If the leaves were big enough to be cored (Figs. 2A and 2B), leaf disks were removed (7 mm diameter), floated in water for 30 min and subsequently transferred to tubes containing 6 ml distilled water. If the plants were too small to be cored (Fig. 3C, Fig. 4A and Fig. S6B), whole leaves were harvested and their fresh weight determined. Plant material was then dipped into water to remove soil and subsequently transferred to tubes containing 6 ml distilled water. Conductivity of the solution was determined with an Orion Conductivity Meter at the indicated time points. Means and standard errors were calculated from four replicate measurements per genotype per experiment. For each measurement, we used six leaf disks or 40-50 mg fresh weight, which equaled 4-6 leaves. The entire experiment was performed three times.

**Demonstration of lethality of AtMC1-ΔN in lsd1**

We crossed *lsd1* (female) to two different *AtMC1-ΔN* overexpressing lines (1.3 and 2.8). F2 plants were selected for the presence of the transgene and analyzed for homozygosity of *lsd1*. Among 144 F2 individuals no homozygous *lsd1* plant was recovered.

**Protein analysis**

For the analysis of protein accumulation in Arabidopsis plants, leaf samples were snap frozen in liquid nitrogen and mechanically ground in 250 μl of plant extraction buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% Triton X-100 and 0.1% SDS) supplemented with a 1:100 dilution of plant protease inhibitor cocktail (Sigma-Aldrich). Protein extract was centrifuged 5 minutes at 10,000 x g at 4°C. The supernatants were collected, protein concentration was measured and the protein extracts were boiled on SDS-loading buffer (120 mM Tris, pH 6.8, 50% Glycerol, 6% SDS, 3 mM DTT and 1% Bromophenol blue) and separated on 12% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes by semi-dry western blot. Membranes were first blocked with a 5% milk-Tris Buffer Saline-Tween 20 (TBST) solution and then incubated in a 1% milk-TBST solution containing a 1:5,000 dilution of anti-HA monoclonal antibody (3F10, Roche) or a 1:5 dilution of anti-myc monoclonal antibody (Tissue Culture Facility, UNC). Protein complexes were labeled with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and detected using the Enhanced Chemiluminiscence Reagent (ECL), ECL-Plus or a 2:1 combination of ECL:ECL-Plus (GE Healthcare).
In order to analyze protein accumulation in yeast, EGY48 cells used for the yeast-two-hybrid assay (see below) were grown overnight in a 28°C shaker on 3 ml of selective Drop Out Base (DOB)-glucose selective media lacking tryptophan. The following day, OD_{600} was measured and adjusted to 0.2 in 4 ml of selective media. Cells were allowed to grow to an OD_{600} of 0.4 and were then centrifuged for 10 minutes at 10,000 x g. The pellets were resuspended in 100 μl of MURB buffer (50 mM Na_{2}HPO_{4}, 25 mM MES, pH 7, 3 M Urea, 1% SDS, 10% β-mercaptoethanol and 0.1% Bromophenol Blue) and processed into crude cell extracts by boiling. The extracts were subjected to immunoblot analysis as described above using anti-HA antisera.

**Co-Immunoprecipitation Assays**

Whole *N. benthamiana* leaves (approx 1 g each) transiently expressing Dex-AtMC1-HA + 35S::p19 + empty vector filler (OD_{600} 0.2:0.2:0.8), pLSD1::LSD1-myc + 35S::p19 + empty vector filler (OD_{600} 0.8:0.2:0.2), Dex-AtMC1-ΔN-HA + 35S::p19 + empty vector filler (OD_{600} 0.2:0.2:0.8), Dex-AtMC1-ΔN-HA + pLSD1::LSD1-myc + 35S::p19 (OD_{600} 0.2:0.8:0.2), Dex-AtMC1-ΔN-HA + pLSD1::LSD1-myc + 35S::p19 + empty vector filler (OD_{600} 0.2:0.2:0.8), Dex-AtMC2-HA + pLSD1::LSD1-myc + 35S::p19 (OD_{600} 0.2:0.8:0.2) and Dex-AtMC1-HA + Est-AtMC2-myc + 35S::p19 (OD_{600} 0.2:0.8:0.2) were frozen in liquid nitrogen. Samples were ground on ice in 3 ml co-immunoprecipitation extraction buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 100 μM ZnSO_{4}) supplemented with 1 mM DTT and 1:100 dilution of plant protease inhibitor cocktail (Sigma-Aldrich). Protein extracts were passed through a miracloth filter (Calbiochem) and centrifuged 15 minutes at 20,000 x g at 4°C. Supernatants were collected for further processing. Protein concentration was adjusted to 2 mg/ml. A 200 μl aliquot of this extract was immediately boiled in SDS-loading buffer (input). A second, 1 ml aliquot was incubated with anti-myc-conjugated magnetic beads (MACS) for 2 h at 4°C with constant rotation. The mixture was passed through a μ Column (MACS). Matrixes were first washed with Lysis Buffer (MACS), followed by three washes with co-immunoprecipitation extraction buffer before adding the bead/protein mix. Finally the columns were washed 4 times with extraction buffer (containing 250 mM NaCl and supplemented with 1 mM DTT) and then with Wash Buffer 2 (MACS) and the fraction of proteins bound to the beads were eluted with Elution Buffer (MACS) pre-heated to 95°C. 40 μg of input and 20 μl of eluate (16x concentrated compared to input) were separated on a SDS-PAGE gel and immunoblotted as described above.

Co-immunoprecipitation assays in Arabidopsis were performed with F1 plant populations originated from crosses between *lsd1 atmc1 pLSD1::LSD1-myc* and *lsd1 atmc1 Dex-AtMC1-HA transgenic lines. lsd1 atmc1 Dex-AtMC1-HA plants were used as a negative control. The same protocol as described above for *N. benthamiana* was followed with the following changes: 200 mg of leaves were used per extraction; centrifugation was done at 4,000 x g; after adding the bead/protein mix, columns were washed three times with Wash Buffer 1 (MACS) adjusted to 300 mM NaCl; 65 μg of input was loaded on an SDS-PAGE gel when immunoblotted with anti-myc.

**Pathogen Strains, Inoculation and Growth quantification**
Hyaloperonospora arabidopsidis isolate Emwa1 and Noco2 were propagated on the susceptible Arabidopsis ecotypes Ws-2 and Col-0, respectively (S14). Conidia were suspended in water at a concentration of 50,000 spores/ml and spray-inoculated onto 11-16 day week old plants (S9). Inoculated plants were kept covered with a lid to increase humidity and grown at 19°C with a 9h light period. To test the growth of Pseudomonas syringae pv. tomato DC3000(avrRpm1) dip inoculations were performed as previously described (S15, S16).

Yeast-Two-Hybrid Analysis

Yeast-two-hybrid analysis was performed using the LexA system. Full length and deletion variants of AtMC1 and AtMC2 were cloned into a Gateway-compatible version of pEG202 (bait) and transformed into yeast RFY206 cells. Full length LSD1 and its deletion variants were cloned into a Gateway compatible version of pJG4-5 (prey) and transformed into yeast EGY48 cells. Mating was performed on 96-well plates overnight in a 28°C shaker. Colonies containing both plasmids were selected on Drop Out Base (DOB)-glucose selection media lacking uracil, tryptophan and histidine (-U, -W, -H). The interactions were analyzed qualitatively by restreaking the yeast onto DOB-galactose (2%) /raffinose (1%) (-U, -W, -H) media containing 160 µg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). The plates were incubated 1 or 2 days at 28°C. As a negative control, DOB-glucose (-U, -W, -H) X-Gal plates were used. The interactions were considered positive if blue color developed on DOB-galactose/raffinose but not on DOB-glucose X-Gal plates. Quantitative β-Galactosidase colorimetric reporter activity assays were performed with a plate reader (Tecan) as described (S17).

Statistical analysis of data

Analysis of variance (ANOVA) was performed on the data, using the JMP program (SAS/JMP software; Version 7.0.1; SAS Institute). The Tukey's honestly significant difference (HSD) test (P<0.05) was applied to separate means.
**Figure S1: Yeast-two-hybrid analysis.**

**A)** Sequence alignment of the CxxCRxxLMYxxGASxVxxCxxC zinc-finger domain of LSD1 and the type I metacaspases AtMC1, AtMC2 and AtMC3 ("Z1" in **B**). Note that the amino acid sequence of the AtMC3 Zinc finger domain is significantly divergent and AtMC3 was thus not considered in this work. **B)** Scheme of AtMC1, AtMC2 and LSD1 proteins (not to scale). Bars with numbers 1-11 inscribed in circles represent different fragments used for the yeast-two-hybrid assay; Z1, Z2 and Z3, LSD1-like zinc finger domains; PP and PPP, proline-rich region; p20 and p10, putative caspase-like catalytic domains; numbered H and C, putative catalytic
histidine and cysteines, present in the caspase-hemoglobinase fold (S18). *atmc1* and *atmc2* indicate the T-DNA insertion sites. C) Qualitative and D) quantitative analysis of the yeast-two-hybrid interactions between different combinations of AtMC1, AtMC2 and LSD1 shown in (B) where they are labeled 1-11. Pairwise interactions between LSD1 and AtMC1 and AtMC2 were tested using a LexA-based yeast-two-hybrid system. Full length LSD1 and its deletion variants (7-11 in B) were used as a prey. Full length AtMC1, or AtMC2 and their derivatives (1-6 in B) were used as bait. LSD1 can homodimerize in this assay (S3). C) Pictures of yeast growing on selective X-Gal media were taken 1 or 2 days after plating (dap). D) β-Galactosidase colorimetric reporter activity assays for quantitative analysis of the yeast-two-hybrid interactions. As expected, LSD1 interacted strongly with itself (246 ± 22 Miller units, mu). Moreover, LSD1 interacted with AtMC1 (126 ± 11 mu). Both the second and third zinc-fingers of LSD1 were required for the LSD1-AtMC1 interaction, but neither of them alone was sufficient. The same requirement was reported for the interaction between LSD1 and AtbZIP10, a transcription factor that acts antagonistically to LSD1 (S3). Interestingly, AtMC1 homodimerizes in this assay via its predicted prodomain (49 ± 12 mu). Both the predicted AtMC1 and AtMC2 prodomains interacted weakly (<15 mu), though above background, with full length LSD1 and truncated LSD1 derivatives containing the second and third zinc fingers. Conversely, the truncated forms of AtMC1 and AtMC2 lacking the predicted prodomain did not interact with each other or with any of the LSD1 variants (shown in C). EV, empty vector. CPRG, Chlorophenol red-β-D-galactopyranoside substrate. Error bars represent 2x standard error. E) Proteins that do not interact in the yeast-two-hybrid assay are expressed in yeast (AtMC1-ΔN, AtMC2 and AtMC2-ΔN). Protein was extracted from EGY48 yeast cells and westerns were immunoblotted using the anti-HA antibody. The arrowhead indicates the band corresponding to AtMC1. Note that the proteins analyzed contain a translational fusion present in the pJG4-5 vector that increases their apparent molecular weight by 10.6 kDa.
Figure S2: AtMC1 interacts with LSD1 via its putative prodomain. AtMC2 does not interact with LSD1 or AtMC1.

Protein extracts from *Nicotiana benthamiana* leaves transiently expressing **A)** full length Dex-AtMC1-HA, Dex-AtMC1-ΔN-HA lacking the putative prodomain or a mixture of pLSD1::LSD1-myc with either Dex-AtMC1-HA or Dex-AtMC1-ΔN-HA, **B)** Dex-AtMC2-HA, Dex-AtMC2-ΔN-HA or a mixture of pLSD1::LSD1-myc with either Dex-AtMC2-HA or Dex-AtMC2-ΔN-HA and **C)** Dex-AtMC1-HA or a mixture of Dex-AtMC1-HA with Est-AtMC2-myc, were subjected to immunoprecipitation using anti-myc-coupled magnetic beads. Forty μg of crude extract (input) and a 16x concentrated fraction containing the eluate (elution) were analyzed by SDS-PAGE/anti-HA or anti-myc immunoblot.
Figure S3: Developmental and stress-related phenotypes.

A) Pictures of eight-week-old Col-0 wild type and the mutants used in this study. Plants were grown in a growth chamber under short-day conditions during 15 days and then transferred to the greenhouse and grown under long-day conditions for 6 more weeks. B) 3,3'-Diaminobenzidine (DAB) stains of leaves from 4-week-old Col-0 and the mutants used in this study 3 days after infiltration with Xanthine/Xanthine Oxidase (X/XO).
Figure S4: AtMC1 is expressed in spatially restricted zones at lsd1 runaway cell death sites.

Four-week-old plants of the genotypes lsd1[pAtMC1::GUS] (A) and lsd1[pAtMC2::GUS] (B) were sprayed with 150 μM BTH. Leaves were harvested at 0 and 1 day after treatment (dpi), stained with Magenta-Gluc and analyzed under bright field and UV-epifluorescence to detect induction of expression and auto-fluorescent secondary metabolites that accumulate upon cell death (S19), respectively. Scalebar, 100 μm; BF, bright field; Epi, epifluorescence, inverted; merged, BF and Epi pictures merged.
Figure S5: *AtMC1* is expressed in spatially restricted zones at HR sites

A) Two-week-old Col-0, *pAtMC1::GUS* or *pAtMC2::GUS* plants were inoculated with 50,000 spores/ml of the *Hpa* isolate Emwa1 to trigger RPP4-mediated HR and disease resistance. At 2 dpi, primary leaves were harvested and double stained with Magenta-Gluc and Aniline Blue, which stains callose deposits and is a proxy for HR ([S11], [S19]). Epifluorescence was used to visualize aniline blue stained callose deposition and *Hpa* hyphae. Scalebar, 100 μm; BF, bright field; Epi, epifluorescence (inverted for Col-0); merged, BF and Epi pictures merged. As
expected, infected Col-0 exhibited HR at sites of autofluorescence (top row). \( p_{AtMC1::GUS} \) plants displayed intense Magenta-Gluc staining in a limited spatial zone of several cells directly surrounding HR sites (middle row). \( p_{AtMC2::GUS} \)-driven GUS-expression was also present around infection sites, but was spatially more diffuse than that of \( p_{AtMC1::GUS} \) (lower row). B) Two-week-old Col-0, \( p_{AtMC1::GUS} \) or \( p_{AtMC2::GUS} \) plants were inoculated with 50,000 spores/ml of the virulent \( Hpa \) isolate Noco2. At 1 and 3 dpi, primary leaves were harvested and double stained with Magenta-Gluc and Aniline Blue. Scalebar, 100 \( \mu \)m. There is no induced expression of \( AtMC1 \) around the freely elongating hyphae (blue stained) (middle row), nor does this pathogen isolate alter the weak expression of \( AtMC2 \) (bottom row). C) \( AtMC1 \) expression is induced after inoculation with hemibiotrophic \( Pseudomonas syringae \) pv. \( tomato \) strain DC3000(\( avrRpm1 \)) to trigger RPM1-mediated HR and disease resistance. 16-day-old \( p_{AtMC1::GUS} \) or \( p_{AtMC2::GUS} \) plants were vacuum infiltrated with 500,000 cfu/ml of \( Pto \) DC3000(\( avrRpm1 \)). Primary and secondary leaves were harvested 12 hours later and double stained with Magenta-Gluc and Trypan Blue. Scalebar, 200 \( \mu \)m. \( AtMC1 \) was expressed adjacent to HR sites following recognition \( Pto \) DC3000(\( avrRpm1 \)) (left). \( AtMC2 \) expression was diffuse and excluded from HR sites (right).
Figure S6: Full-length AtMC2 is a negative regulator of cell death.

Seedlings of the depicted genotypes were sprayed with 1 μM Dex 6, 11 and 16 days after germination. CACA, denotes constructs where both putative catalytically active cysteine residues (C135A C256A) have been mutated to alanine. A, C, D and F) AtMC2 protein accumulation in different transgenic lines. One day after the last treatment with Dex, tissue was harvested and total protein isolated. Westerns were immunoblotted with the anti-HA antibody. B) Full-length AtMC2 partially inhibits lsd1 runaway cell death. One day after the last treatment with Dex, plants were sprayed with 150 μM BTH. Tissue was harvested at 43 hpt and processed
for conductivity measurements. Blue lines: Dex-treated genotypes; Yellow lines: non Dex-treated genotypes. Error bars represent 2 x standard error. Letters a - f represent experimental groups with significant differences ($P < 0.05$, Tukey’s HSD test). The experiment was repeated twice. hpt, hours after treatment. 

E) Ten hours after the last treatment with Dex plants were vacuum infiltrated with 500,000 cfu/ml of $Pto$ DC3000($avrRpm1$). Twelve hours later, plants were harvested and stained with Trypan Blue to visualize cell death. To quantify cell death, all dead cells in one field of vision (10x magnification) were counted. Average and 2 x standard error were calculated from 20 leaves per genotype and treatment.
Figure S7: AtMC2 does not transcriptionally regulate AtMC1

An *lsd1 pAtMC1::GUS* line was crossed to *lsd1 AtMC2-ΔN* line 2.8. F1 seedlings and seedlings of the *lsd1 pAtMC1::GUS* parental control line were treated with 1 μM Dex 6, 11 and 16 days after germination. One day after the last treatment with Dex, plants were treated with 150 μM BTH. Leaves were harvested 1 day after BTH treatment, stained with Magenta-Gluc and analyzed under bright field and UV-epifluorescence to detect induction of AtMC1 expression and auto-fluorescent secondary metabolites that accumulate upon cell death (*S19*), respectively. Scalebar, 100 μm; BF, bright field; Epi, epifluorescence; merged, BF and Epi pictures merged. Note the suppression of autofluorescence, but not Magenta-Gluc staining in samples from the F1, confirming that although *pAtMC1::GUS* is still expressed, the wild type AtMC1 activity is suppressed.
References for SOM