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●第62回日本自律神経学会総会/シンポジウム1/生体リズム研究の新展開―時計遺伝子のジェネティクス，エピジェネティクス，そして臨床応用まで―
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概日リズムによる生理機能の制御機構

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キーワード：概日リズム，分子時計，転写
circadian rhythm, molecular clock, transcription

抄録：概日リズムは生理現象の周期を外環境に適応させ維持する機構であり，分子時計と呼ばれる全身の個々の細胞に存在する転写/翻訳に依存したフィードバックループにより制御されている[1]．精神的ストレスや不規則な生活は概日リズムの異常を引き起こすが，このリズムの異常は精神（時差症候群，不眠，うつ），循環器（心筋梗塞，高血圧），呼吸器（喘息）疾患等の自律神経の異常に起因する疾患の病態に関与する[7]．さらに，近代化がもたらす飽食に伴うメタボリック症候群，老齢化と関連する骨粗鬆症や発癌といった現代生活を脅かす疾患が概日リズムと密接に関係していることが明らかになっている[3]．本総説は，分子時計の制御機構及び分子時計と疾患の関連についての最近の知見を要約する．
（自律神経，47：297～300, 2010）

分子時計の転写制御機構

概日リズムは全身の個々の細胞に存在する転写/翻訳に依存したフィードバックループ（分子時計）により制御されている[2]．特に脊椎動物の分子時計はCLOCK，NPAS2, BMAL1, PER（哺乳動物の場合は，PER1, PER2, 及びPER3が存在する．）及びCRY（哺乳動物の場合は，CRY1及びCRY2が存在する．）と呼ばれる転写因子（時計蛋白質）により構成される約24時間の周期性をもつ転写/翻訳に依存したフィードバックループである（図1）．CLOCK（NPAS2）とBMAL1は二量体を形成し*Per*及び*Cry*の転写を活性化する．PERおよびCRYは翻訳後二量体を形成し，CLOCK（NPAS2）：BMAL1複合体に直接結合しその転写を抑制する．重要なこととして，CLOCK（NPAS2）：BMAL1二量体は，脂肪細胞の分化に関わる*Peroxisome proliferator-activated receptor* α，肝臓における新陳代謝を制御する*Albumin D element-binding protein*，細胞周期制御因子*Wee1*といった様々な遺伝子の転写調節を担う．特に，細胞内の約10%の遺伝子がCLOCK（NPAS2）：BMAL1二量体により転写調節されていることが報告されている[12]ことから，分子時計は様々な遺伝子の転写調節を介して多くの細胞機能を制御していると考えられている．

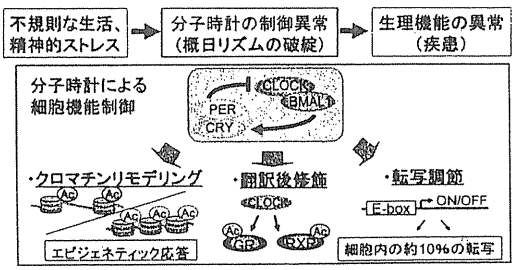

図1　分子時計による細胞機能調節のモデル

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表1　時計遺伝子変異マウスの表現型

遺伝子	概日リズムに関する表現型	概日リズム以外の表現型
CLOCK -del.19 mutant	恒暗条件下において行動リズムの周期の約4時間の延長が観察された後，行動リズムが消失する． 分子時計により制御される遺伝子発現の日周性の消失． 視交叉上核における時計遺伝子発現の光誘導の減少． 視交叉上核のニューロンの神経発火は正常であることから，行動リズムの消失は全身の個々の細胞の分子時計の脱同調に起因すると考えられる．	代謝異常及び糖尿病の発症． 加齢に伴う唾液腺増殖の発症． 躁病の発症．
CLOCK KO	行動リズムは正常． 行動リズムの光同調の異常 分子時計により制御される遺伝子発現の日周性の異常．	報告なし．
BMAL1（MOP3）KO	恒暗条件下における行動リズムの消失． 活動量の減少． 分子時計により制御される遺伝子発現の日周性の消失．	老化の促進による寿命の短縮． 末梢血，脾臓，骨髄におけるB細胞の減少． BMAL1ノックアウトマウス胚由来線維芽細胞は脂肪細胞への分化能を消失．
NPAS2（MOP4）KO	恒暗条件下及び明暗条件下で正常な行動リズムを示す．	長期記憶の異常． ノンレム睡眠の異常．
CLOCK：NPAS2 double KO	恒暗条件下の行動リズムの消失．	報告なし．
PER1 KO	恒暗条件下の行動リズムの周期の短縮． 行動の光同調の異常（光による行動周期の前進が観察されない．） PER1：PER3 double KOマウスは，PER1 KOマウスと同じ表現型を示す．	報告なし．
PER2 KO	恒暗条件下において行動リズムの周期性の短縮が観察された後，行動リズムが消失する． 行動の光同調の異常（光による行動周期の後進が観察されない．） PER2：PER3 double KOマウスは，PER2 KOマウスと同じ表現型を示す．	リンパ腫の自然発症率の増加． ガンマ線照射後の癌発症率の増加．
PER3 KO	恒暗条件下及び明暗条件下で正常な行動リズムを示す．	報告なし．
PER1：PER2 double KO	恒暗条件下の行動リズムの消失． PER1：PER2：PER3 triple KOマウスは，PER1：PER2 double KOマウスと同じ表現型を示す．	報告なし．
CRY1 KO	恒暗条件下の行動リズムの周期の短縮．	報告なし．
CRY2 KO	恒暗条件下の行動リズムの周期の延長．	報告なし．
CRY1：CRY2 double KO	恒暗条件下の行動リズムの消失．	野生型マウスと同様のDNA損傷応答を示す．
CRY1：CRY2：p53 triple KO	報告なし．	p53変異マウスの腫瘍形成及び寿命の短縮の表現型が緩和する．
CRY1：PER2 double KO	恒暗条件下の行動リズムの消失．	報告なし．
CRY2：PER2 double KO	恒暗条件下及び明暗条件下で正常な行動リズムを示す．	報告なし．

分子時計制御における時計蛋白質の翻訳後修飾の役割

本来，転写及び翻訳は24時間よりもはるかに短い周期で行われる．従って，分子時計の周期を約24時間に維持するための細胞内機構の存在が考えられる．近年この機構に時計蛋白質の酵素活性及び翻訳後修飾の制御が重要な役割を担うことが報告されている[5]．例えば，時計蛋白質CRYは翻訳後リン酸化修飾され分解されるため，翻訳と細胞内蓄積には時間ラグが生じる．このラグは分子時計に約24時間の周期性を与えるために重要な過程である．

リン酸化以外の分子時計制御に関わる翻訳後修飾として，Sumo化及びアセチル化修飾が報告されている．BMAL1は時間依存的にSumo化修飾され，このSumo化修飾はBMAL1の安定性を制御する．CLOCKは長い間BMAL1と共同的に働く転写因子と考えられていた．近年，CLOCKがヒストンアセチルトランスフェラーゼ（HAT）活性を有することが報告され，CLOCKが酵素であることが明らかにされている[13]．ヒストンのアセチル化は遺伝子の転写活性に関与することが広く知られているが，CLOCKは自身のHAT活性により時間依存的にヒストンをアセチル化し，CLOCK：BMAL1二量体により転写活性化される遺伝子の時間依存的な転写制御を行う．また，CLOCKがそのHAT活性によりBMAL1を時間依存的にアセチル化すること及びこのCLOCKによるBMAL1のアセチル化は転写抑制因子CRYのCLOCK：BMAL1二量体へのリクルートメントを促進することが報告されている[6]．従って，CLOCKのHAT活性は分子時計のターゲット遺伝子の転写活性と転写抑制の両方の過程で重要な役割を担っている．興味深いことに，NAD^+依存的な脱アセチル化酵素SIRT1が時間依存的にBMAL1を脱アセチル化しBMAL1のアセチル化に日周性を与えることが報告されている[10]．SIRT1はDNA損傷応答や脂肪代謝制御等の多くの生理機能を制御することが知られている．従って，CLOCK及びSIRT1による時計蛋白質の翻訳後修飾という知見は分子時計が概日リズム以外の細胞機能をその調節因子の翻訳後修飾を介して制御することを強く示唆している．実際に，最近時計蛋白質CLOCKがアセチル化を介して糖代謝制御に関わるグルココルチコイドレセプター（GR）を機能調節することが報告されている[9]．

分子時計と疾患

躁鬱病の患者には睡眠/覚醒，体温，ホルモンの放出等の生理現象の概日性周期の異常が頻繁に観察されることが知られているように，自律神経疾患を含む多くの疾患の病態に時間因子が関与することが古くから報告されている．近年，時計蛋白質の変異マウスが概日リズムの異常に加え多くの疾患を発症することが報告され（表1），一部その病態メカニズムに分子時計が関与していることが強く示唆されている．例えば，*Clock*変異マウスが通常より高い頻度で躁病になることが報告されている[13]．また，*Per2*ノックアウトマウスは高頻度でリンパ腫を発症し[4]，*Bmal1*ノックアウトマウスは老化の促進という表現型を示す[8]．さらに，*Clock*変異マウスは代謝異常及び糖尿病を発症することが報告されている[14]．

分子時計は睡眠/覚醒，代謝，細胞周期等の様々な生理機能を制御する遺伝子の転写調節を介して他の細胞機能に影響を与える．また，時計蛋白質CLOCKは自身のHAT活性により，概日リズム以外の細胞機能を制御する蛋白質を翻訳後修飾し機能調節する．さらに，分子時計はCLOCKのHAT活性によりターゲット遺伝子の発現調節領域のクロマチンリモデリングを行うが，これは分子時計が細胞のエピジェネティック応答を担う可能性を示唆する（図1）．分子時計は多くの細胞機能の制御を担っていることから，分子時計の制御異常が直接疾患に関連することが考えられる．従って，分子時計の制御機構の詳細な理解は自律神経疾患をはじめとする病態の解明に新たな視点を与え，さらに分子時計の転写制御を指標とした診断法の開発や時計蛋白質を標的とした創薬に還元されることが期待される．

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Review

A Common Origin: Signaling Similarities in the Regulation of the Circadian Clock and DNA Damage Responses

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Circadian clocks are intrinsic, time-tracking systems that endow organisms with a survival advantage. Studies of animal models and human tumor samples have revealed that the disruption of circadian rhythms is an important endogenous factor that can contribute to mammalian cancer development. The core of the circadian clock mechanism is a cell-autonomous and self-sustained oscillator system mediated by a transcription/translation-based negative feedback loop that relies on positive and negative elements. Recent studies have implicated these core circadian components in the regulation of both the cell cycle and DNA damage responses (DDR). Indeed, the circadian feedback loop controls the timing of cell proliferation by regulating the expression of key cell cycle genes. Conversely, several intracellular signaling cascades and post-translational modifications that play important roles in the cell cycle and DDR are also essential for circadian clock regulation. Importantly, alteration of a cell's reduction–oxidation (redox) state triggers the transduction of photic signals that regulate circadian clock gene transcription, suggesting that cellular responses to photo-oxidative stress may have been the evolutionary origin of the circadian clock. This review describes selected regulatory aspects of circadian machinery that are evidence of a molecular link between the circadian clock and DDR, focusing particularly on the signaling cascades involved in the light entrainment of the zebrafish circadian clock.

Key words circadian clock; DNA damage response; reduction–oxidation; zebrafish

INTRODUCTION

From bacteria to humans, almost all organisms can adapt the timing of their physiology to the cyclic changes of their environment, thanks to a naturally-selected intrinsic time-keeping system called the circadian clock.[1] The circadian clock enhances the physiological efficiency and survival of an organism by organizing its behavior and body functions.[2,3] During the circadian day, the organism's physiology is given over to catabolic processes, whereas the anabolic functions of growth, repair and consolidation occur at night. To achieve this schedule in mammals, the circadian clock regulates a number of physiological functions, including sleep and wakefulness, food intake, body temperature, cardiovascular and renal activity, hormone production, hepatic metabolism and immune responses.[4,5] Accordingly, disruption of the circadian clock in humans has been linked to profound effects on health, including insomnia, stomach ailments, depression and cancer.[4,5]

In most organisms, the molecular mechanisms underlying the establishment and maintenance of biological rhythms comprise interconnected transcription–translation feedback loops in which some clock factors repress their own transcription once they have attained critical levels.[2,3] These oscillators have the property of being endogenous and cell-autonomous systems that maintain their rhythm in the absence of external time cues.[6] Both vertebrates and invertebrates have circadian oscillators scattered throughout their bodies.[7,8] In mammals, the circadian system is composed of both central and peripheral oscillators.[7] The mammalian central clock is located in the suprachiasmatic nucleus (SCN) within the anterior hypothalamus of the brain.[9] This central clock acts as a coordinator and provides time signals via both neural and humoral routes that entrain independent peripheral clocks. Dysfunction of the central clock does not inactivate the peripheral clocks but instead causes individual peripheral oscillators to become temporally uncoupled.[10—12]

To guarantee that an organism's behavior remains tied to the rhythms of its environment, the circadian clock must be able to reset itself in response to environmental cues.[9,13,14] The main environmental stimulus for organisms is light, which is provided in day-night cycles. Mammals have no photoreceptors in peripheral tissues,[15] so that the effect of light on peripheral clocks is indirect.[13] For the mammalian clock, the SCN integrates photic cues from the retina and uses neural and humoral signals to transmit this information to peripheral clocks, synchronizing them.[16—18] This communication between the central and peripheral clocks results in the seamless regulation of fundamental physiological functions.[4,8] Interestingly, peripheral clocks can also respond directly to SCN-independent signals such as feeding and temperature change.[19,20] However, the physiological role of SCN-independent responses of peripheral clocks is not yet fully understood. A recent study has reported that DNA damage can also act as a resetting cue for the circadian clock in mammalian peripheral cells,[21] and other findings support a major role for the circadian machinery in cellular stress responses.[22] These data point to an intriguing link between the circadian clock and cellular stress responses, and it is the purpose of this review to summarize the evidence and explore the implications of such a link.

THE PHYSIOLOGICAL FUNCTIONS OF CORE CIRCADIAN REGULATORS

The core of the clock mechanism in *Drosophila*, *Neurospora* and mammals is a transcription/translation-based negative feedback loop that relies on positive and negative

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oscillator elements. In vertebrates, three basic helix–loop–helix Perid-Aryl hydrocardon receptor nuclear translocator-Single-minded (PAS) (PER-ARNT-SIM) domain-containing transcription factors, called CLOCK, NPAS2 and BMAL, constitute the positive elements.[3,23] CLOCK or NPAS2 heterodimerizes with BMAL to form an transcriptionally active complex that binds to E-box elements (CACGTG) present in the promoters of members of the *Period* (*Per*) and *Cryptochrome* (*Cry*) gene families (Fig. 1). Once the PER and CRY proteins have been translated, they form heterodimers that can then translocate to the nucleus to repress CLOCK (NPAS2):BMAL-mediated transcription through direct protein–protein interaction. Importantly, when active, the CLOCK (NPAS2):BMAL complex stimulates the transcription of many other clock-controlled genes. These genes in turn influence functions external to the oscillatory mechanism itself and mediate the "output" function of the clock.[24] This accounts in part for the presence of circadian rhythms in a variety of physiological processes.

A group of eight proteins comprises the basic sprockets of the molecular wheel that controls the mammalian circadian clock: PER1, PER2, PER3, CRY1, CRY2, CLOCK, NPAS2 and BMAL1.[3,23] The phenotypes of mice with targeted disruptions of these genes are summarized in Table 1. Studies of these mutant mice have revealed the distinct roles of clock proteins in regulating circadian rhythms as well as direct links between the circadian clock and non-circadian aspects of animal physiology. For example, mice with mutations in the *Per2* and *Bmal1* genes show increased sensitivity to ionizing radiation and a premature aging phenotype, respectively.[25,26] These findings implicate the core circadian machinery in the regulation of DNA damage response (DDR) and the cell cycle.

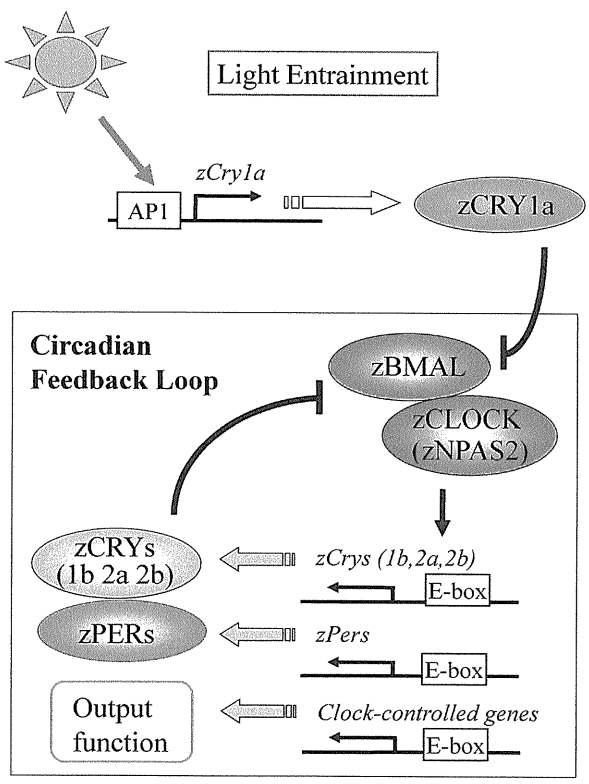

Fig. 1. Key Transcription Factors in the Zebrafish Circadian Feedback Loop

Zebrafish possess an intrinsic circadian oscillator that consists of components similar to those of mammals. CLOCK (NPAS2) and BMAL act as positive elements, whereas CRYs and PERs act as negative regulators. Zebrafish CRY1a, CRY1b, CRY2a, and CRY2b are transcriptional repressors. Expression of *zCry1a* depends on the transcription factor AP-1 and is stimulated by light, whereas *zCry1b*, *zCry2a* and *zCry2b* expression are under the control of the heterodimeric zCLOCK (zNPAS2):zBMAL transcription factor that binds to E-box elements in target gene promoters. Expression of the zPER transcriptional repressors is also stimulated by the zCLOCK (zNPAS2):zBMAL complex. In zebrafish, light-induced zCRY1a inhibits the transcription dependent on zCLOCK (zNPAS2):zBMAL, thereby regulating the light entrainment of the circadian clock.

At the molecular level, the circadian clock controls the timing of cell proliferation by regulating the expression of key cell cycle genes such as *Wee1* and *c-Myc*.[25,27] In addition, PER1 interacts with crucial components of cellular stress response pathways including the ataxia telangiectasia mutated (ATM) and checkpoint kinase 2 (Chk2) proteins.[28] Accordingly, ectopic expression of PER1 or PER2 results in cell growth inhibition, cell cycle arrest, apoptosis, or loss of clonogenic capacity.[28–30] PER1 and PER2 also interact with the androgen receptor (AR) or estrogen receptor (ER), respectively, in that PER1 inhibits AR-dependent transcription and PER2 induces ER degradation.[31,32] These findings are consistent with the idea that clock proteins act as key players in the cell cycle and DDR by interacting directly with and regulating the functions of the proteins mediating these processes.

POSSIBLE CROSSTALK BETWEEN THE CIRCADIAN CLOCK AND CELLULAR PROCESSES THROUGH SHARED POST-TRANSLATIONAL MODIFICATIONS

Post-translational modifications, such as phosphorylation, sumoylation and acetylation, are important modulators of circadian transcription factors and regulate their transcriptional activity, subcellular localization and stability.[6,33] The effects of selected post-translational modifications of various circadian regulators are summarized in Table 2. Below we discuss the role of clock protein acetylation in circadian regulation. The detailed functions of other post-translational modifications of clock proteins have been reviewed elsewhere.[6,33,34]

In mammals, the core circadian regulator CLOCK has intrinsic histone acetyltransferase (HAT) activity[35] that it uses to acetylate its heterodimeric partner BMAL1.[36] This CLOCK-mediated acetylation increases the interaction of the CLOCK:BMAL1 complex with CRY1, providing another level of control in the circadian negative feedback loop.[36] BMAL1 is deacetylated by SIRT1, a nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase (HDAC).[37] Accordingly, BMAL1 acetylation is significantly increased and only mildly rhythmic in livers of liver-specific *Sirt1*-deficient mice.[37] SIRT1 also deacetylates PER2, giving SIRT1 an additional function in circadian transcription regulation.[38] The finding that CLOCK can acetylate non-histone substrates such as BMAL1 has sparked a search for other cellular targets. CLOCK interacts directly with the nuclear receptors retinoic acid receptor (RAR)α and retinoid X receptor (RXR)α through CLOCK's putative nuclear receptor interaction domain (NRID),[39] suggesting that CLOCK may acetylate nuclear receptors. Similarly, CLOCK interacts with and acetylates the glucocorticoid receptor, negatively regulating its transactivation capacity.[40] Thus, in addition to its ef-

Table 1. Phenotypes of Mutant Mice Disrupted in Circadian Clock Genes

Name	Mutation	Circadian phenotype	Non-circadian phenotype
CLOCK -del.19 mutant	Deletion of exon 19 in murine CLOCK gene.[89] Produces a mutant CLOCK protein that functions as a dominant negative regulator.[35]	Circadian period extended by 4h, followed by a complete loss of circadian rhythmicity in DD.[a),89] Displays reduced levels and non-cycling of expression of clock-controlled genes.[89] Displays reduced light induction of immediate early genes in SCN.[90] Shows normal spontaneous firing rhythms of SCN neurons, suggesting that loss of circadian rhythmicity may be due to uncoupling of oscillators.[91]	Diurnal feeding rhythm attenuated.[92] Develops obesity and metabolic syndrome.[92] Develops salivary gland hyperplasia with age.[93] Displays mania-like behavior, including hyperactivity, decreased sleep, lowered depression-like behavior, and lower anxiety.[94]
CLOCK KO	Knockout mutation.[95]	Exhibits almost normal circadian patterns of behavior.[95] Shows altered responses to light, with reduced phase delays and exaggerated phase advances.[95] Displays altered clock-controlled gene expression.[95]	None reported
BMAL1 (MOP3) KO	Knockout mutation.[96]	Displays immediate and complete loss of circadian rhythmicity in DD.[96] Displays altered distribution of activity during LD[a)] and reduced total activity.[96] Shows reduced levels and non-cycling of expression of clock-controlled genes.[96]	Reduced life span with symptoms of premature aging.[26] Displays reduced levels of B cells in the peripheral blood, spleen and bone marrow.[97] BMAL1-deficient embryonic fibroblasts cannot differentiate into adipocytes.[98]
NPAS2 (MOP4) KO	Knockout mutation.[23]	Exhibits almost normal circadian patterns of behavior.[23] Has a slightly shortened circadian period and an altered response to perturbations in the LD cycle.[23]	Displays deficits in the long-term memory arm of the cued and contextual fear task.[99] Displays defect in the homeostatic regulation of non-rapid eye movement sleep time.[100]
CLOCK; NPAS2 double KO	Double knockout.[23]	Displays immediate and complete loss of circadian rhythmicity in DD.[23]	None reported
PER1 KO	Knockout mutation.[101]	Displays a shorter circadian period in DD.[101] Exhibits a defect in circadian clock resetting (unable to advance the clock).[102] Per1; Per3 KO has the same phenotype.[103]	None reported
PER2 KO	Knockout mutation.[103]	Displays a shorter circadian period followed by a loss of circadian rhythmicity in DD.[103] Exhibits a defect in circadian clock resetting (unable to delay the clock).[102] Per2 mutant; Per3 KO has the same phenotype.[103]	Is cancer-prone and shows increased sensitivity to γ-irradiation.[25]
PER3 KO	Knockout mutation.[103]	Has normal circadian pattern of behavior.[103]	None reported
PER1; PER2 double KO	Double knockout.[103]	Displays a complete loss of circadian rhythmicity in DD.[103] PER1; PER2; PER3 triple KO has the same phenotype.[103]	None reported
CRY1 KO	Knockout mutant.[104]	Exhibits accelerated free-running periodicity of locomotor activity.[104]	None reported
CRY2 KO	Knockout mutant.[104]	Exhibits delayed free-running periodicity of locomotor activity.[104]	None reported
CRY1; CRY2 double KO	Double knockout.[104]	CRY1; CRY2 double KO displays immediate and complete loss of circadian rhythmicity in DD.[104]	Normal genotoxic stress-induced morbidity and mortality.[105]
CRY1; CRY2; p53 triple KO	Triple knockout.[106]	None reported	Shows delayed onset of cancer and extended median lifespan upon genotoxic stress compared to mice lacking only p53.[106]
CRY1; PER2 double KO	Double knockout.[107]	Displays an immediate loss of circadian rhythmicity in DD.[107] Normal circadian behavior in LD.[107]	None reported
CRY2; PER2 double KO	Double knockout.[107]	Displays normal circadian behavior in LD and DD.[107]	None reported

a) DD, dark–dark cycle, *i.e.* constant darkness. LD, light–dark cycle.

Table 2. Post-Translational Modifications of Murine Circadian Clock Proteins

Protein	Modification (site)	Regulator	Effect/function
CLOCK	Phosphorylation (unknown)	BMAL1 induces phosphorylation, whereas CRYs induce unphosphorylated form.	Phosphorylated CLOCK is transcriptionally active[108,109] and localized in the nucleus.[110] Phosphorylation may regulate CLOCK's subcellular localization.
	Phosphorylation (Ser-Pro-rich region)	H1 kinase	Unknown.[111]
	Phosphorylation (Ser38 and Ser42)	Unknown	Prevents CLOCK:BMAL1 complex from binding to E-box, thus inhibiting CLOCK:BMAL1-dependent transcription.[34]
	Phosphorylation (Ser427)	GSK-3β	Induces CLOCK degradation.[112] CIPC induces phosphorylation.[34]
	Phosphorylation (unknown)	PKG	Unknown.[113]
	Phosphorylation (unknown)	PKC	PKC-mediated phosphorylation stimulates CLOCK: BMAL1-dependent transcription.[114]
BMAL1	Phosphorylation (unknown)	CLOCK induces phosphorylation, whereas CRYs induce unphosphorylated form.	Phosphorylated BMAL1 is transcriptionally active.[108,109]
	Phosphorylation (unknown)	Casein kinase 1 (CK1)	CKI-mediated phosphorylation stimulates CLOCK: BMAL1-dependent transcription.[115]
	Phosphorylation (Ser-527, Thr-534, Ser-599)	MAPK/ERK	Thr-534 phosphorylation inhibits CLOCK:BMAL1 dependent transcription. Functions of Ser-527 and Ser-599 phosphorylations are unknown.[116]
	Phosphorylation (Ser-90)	Casein kinase 2 (CK2)	Required for BMAL1 nuclear translocation.[43]
	Sumoylation (Lys-259)	CLOCK induces Sumoylation.	Regulates BMAL1 protein half-life.[117]
	Acetylation (Lys-537)	CLOCK acetylates BMAL1, whereas SIRT1 deacetylates it.	CLOCK-mediated BMAL1 acetylation facilitates CRY recruitment to the CLOCK:BMAL1 complex.[36,37]
PER1	Phosphorylation (amino acids 902—916)	CK1	Masks PER1 nuclear localization signal, inducing PER1 cytoplasmic localization.[118]
	Phosphorylation (amino acids 653—663)	CK1	Required for nuclear translocation of PER1.[119]
PER2	Phosphorylation (β-TrCP recognition motif)	CK1	Required for TrCP binding and subsequent PER2 degradation.[120]
	Phosphorylation (unknown)	GSK-3β	Induces nuclear localization of PER2.[48]
	Phosphorylation (Ser-10, Thr-12, Ser-13, and Thr-15)	CK2	Stabilizes PER2.[42]
	Phosphorylation (Ser-53)	CK2	Induces PER2 degradation.[44]
	Acetylation (unknown)	Acetylase is unknown. SIRT1 deacetylates PER2.	Deacetylation of PER2 by SIRT1 induces PER2 degradation.[38]
PER3	Phosphorylation (amino acids 613—626)	CK1	Induces PER3 degradation and nuclear translocation.[121]
CRY1	Phosphorylation (unknown)	CK1	Function unknown. PER acts as a scaffold that brings CK1 and CRY into close proximity.[115]
	Phosphorylation (Ser247)	MAPK/ERK	Reduces transcriptional inhibition activity.[122]
CRY2	Phosphorylation (Ser265)	MAPK/ERK	Reduces transcriptional inhibition activity.[122]
	Phosphorylation (Ser557)	MAPK/ERK (MAPK/ERK phosphorylates the Ser-557 of CRY2 *in vitro*, but it is unlikely to contribute to this phosphorylation *in vivo*.)	Function unknown but phospho-Ser-557-CRY2 is localized specifically in the nucleus and displays robust circadian variation.[51,122]
	Phosphorylation (Ser553)	GSK-3β	Induces degradation.[51] GSK-3β induces this phosphorylation only if Ser-557 is also phosphorylated.[51]

fects on circadian clock elements, CLOCK targets key components of the cell cycle machinery.

It is well established that post-translational modifications are vital for the regulation of the cell cycle and DDR.[41] SIRT1[37,38] and casein kinase 2 (CK2),[42—44] already identified as responsible for post-translational modifications of clock proteins, have also been implicated in post-translational modifications of proteins such as p53, FoxO, and E-cadherin that are involved in cellular metabolism, the cell cycle, and DDR.[45,46] These findings support the hypothesis that the circadian clock may be linked to other cellular processes through shared post-translational modifications.

POSSIBLE CROSSTALK BETWEEN THE CIRCADIAN CLOCK AND CELLULAR PROCESSES THROUGH SHARED INTRACELLULAR SIGNALING CASCADES

Vertebrate circadian feedback loops are affected by an enormous variety of stimuli known to induce intracellular signaling pathways, including pathways involving protein kinase C (PKC), glucocorticoid, Wnt, tumor growth factor (TGF)-β/activin, and mitogen-activated protein kinases (MAPKs).[47—50] Conversely, several studies have demonstrated at a molecular level how these intracellular signaling mediators contribute to circadian regulation. For example, in mammals, glycogen synthase kinase-3β (GSK-3β), the key kinase regulating the Wnt signaling pathway, phosphorylates CRY2 and PER2 and thereby controls CRY2 protein stability and PER2 subcellular localization.[48,51] Another example is the stimulation of *Dec1* gene expression that is induced by the TGF-β/activin signaling pathway during alkaline shock-induced entrainment of mammalian peripheral clocks.[49] The signaling pathways involving these mediators play essential roles in development, proliferation, DDR, and cell death processes, suggesting that these processes and the circadian clock are separate nodes of a common regulatory network. In support of this idea, a recent study in zebrafish has revealed that the same MAPK signaling cascades are involved in both DDR and the light-dependent pathways responsible for entrainment of the circadian clock[52] (see below).

ZEBRAFISH AS A MODEL SYSTEM FOR CIRCADIAN CLOCK STUDY

Zebrafish are a good model organism in which to study the vertebrate circadian clock because the molecular components of the mammalian and zebrafish circadian oscillators are same. Moreover, cell-autonomous circadian oscillators are present throughout the peripheral tissues and organs of zebrafish.[53,54] Importantly, zebrafish peripheral clocks are directly light-responsive.[55] Thus, in zebrafish organs and tissues, an acute light pulse can be used to transactivate clock genes and thus entrain oscillations of clock gene expression to a new light-dark cycle. In addition, cultured lines of embryonal zebrafish cells, which recapitulate most features of the zebrafish clock system, have been established as an attractive vertebrate cell-based model suitable for the examination of the light signaling pathway and its impact on the circadian clock.[56] Studies using these cell lines have revealed critical roles for redox control and MAPK signaling pathways in light-dependent circadian entrainment[57] (Fig. 2).

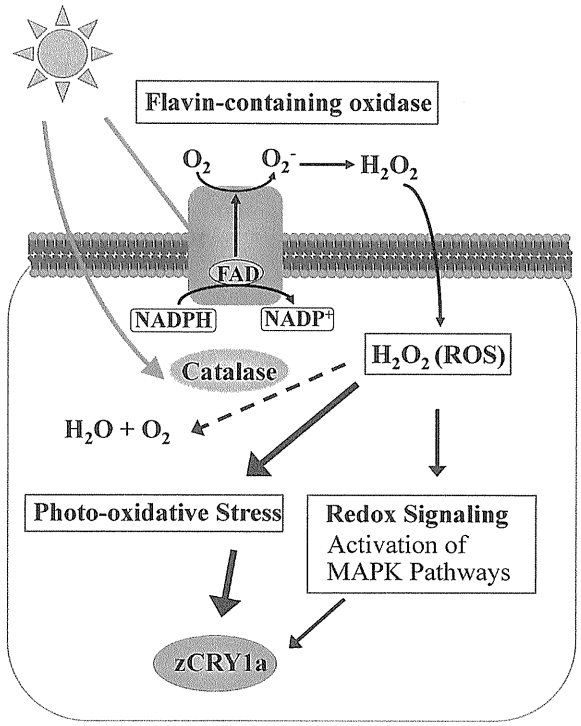

Fig. 2. A Model of a Potential Molecular Mechanism Underlying Light-Dependent Redox Signaling in Zebrafish

In the presence of flavin-containing oxidases, light drives the production of intracellular ROS such as H_2O_2. Excess ROS production has deleterious effects because ROS can react with various cellular targets to cause photo-oxidative stress. However, light-induced ROS can also take on a signaling role by stimulating MAPK pathways that lead to transcriptional activation, including transactivation of the *zCry1a* gene. Importantly, light also increases *catalase* transcription and thus intracellular catalase activity, resulting in degradation of H_2O_2 and decreased photo-oxidative stress. This reduction in ROS also leads to decreased *zCry1a* expression, thus creating a negative feedback loop that directly impinges on the circadian clock.

Other cellular functions, including the cell cycle and DDR, are also directly regulated by light in zebrafish.[52,58,59] The dissection of the light signaling pathways used for circadian entrainment in zebrafish peripheral cells has revealed common mediators also shared by pathways used to regulate the cell cycle and DDR.[52,60]

REDOX SIGNALING IS INVOLVED IN THE LIGHT ENTRAINMENT OF THE ZEBRAFISH PERIPHERAL CLOCK

As a result of whole genome duplication during the evolution of the teleost lineage, the circadian oscillator of zebrafish contains duplications of most clock genes.[53] Seven zebrafish *Cry* genes (*zCry1a*, *1b*, *2a*, *2b*, *3*, *4* and *Dash*) have been cloned.[53,61] Investigation of the *in vitro* functions of these genes has shown that they fall into two groups: one group inhibits CLOCK:BMAL-mediated transcription (repressor type CRYs: zCRY1a, 1b, 2a and 2b), whereas the other group does not inhibit transcription (non-repressor type CRYs: zCRY3, 4 and Dash).[61,62] Despite its structural and functional similarities to the repressor type CRYs, *zCry1a* transcription is quite different from that of the other repressor type CRYs and is strictly light-dependent (Fig. 1, top). *zCry1a* transcription exhibits circadian oscillation in zebrafish cells exposed to a light-dark cycle but this oscillation

dampens quickly after transfer of the cells to constant darkness, indicating that zCRY1a functions only in light-dependent circadian clock regulation.[57,63,64] Indeed, there is evidence that light-induced zCRY1a is a transcriptional repressor essential for the light-dependent entrainment of the circadian clock.[63]

The light-dependent transcription of *zCry1a* is controlled through the production and removal of cellular reactive oxygen species (ROS).[57] ROS were originally thought to act solely as toxic metabolites because they react with components of DNA, proteins and lipids and exert oxidative stress.[65] However, ROS are also ideally suited to be signaling molecules because they are small in size and can easily diffuse short distances within a cell. In addition, mechanisms for ROS production (such as *via* flavin-containing oxidases) and its rapid removal (such as *via* catalase) are present in almost all cell types[65,66] (Fig. 2). In a variety of organisms, light induces ROS production that leads to altered redox status.[65,67] In zebrafish cells, this light-induced redox change stimulates intracellular MAPK signaling that transduces photic signals to *zCry1a* transactivation.[57] Importantly, light also increases intracellular catalase activity by stimulating catalase transcription, an event that occurs after the maximum expression of the *zCry1a* gene has been reached.[57] This increased catalase activity diminishes light-induced cellular ROS levels, resulting in decreased *zCry1a* transcription and creating a negative feedback loop.

The regulation of the circadian clock by redox signaling raises an important issue concerning the identity of the circadian photoreceptor. Because ROS can transduce a photic signal to the circadian machinery, the phototransducing molecules responsible for light-dependent ROS production should function as the circadian photoreceptor in zebrafish cells. We hypothesize that flavin-containing oxidases, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and acyl-CoA oxidase, may be good candidates for the circadian photoreceptors. These enzymes, which produce intracellular ROS, are activated by a plethora of extracellular stimuli, including visible light, wounding, and low and high temperature.[65,67] In addition, we have found that diphenyleneiodonium, an inhibitor of NADPH oxidase, efficiently suppresses light-induced activation of *zCry1a* transcription in cultured zebrafish cells (Uchida *et al.*, unpublished data). Redox signaling also appears to play a significant role in circadian regulation in other organisms. In *Drosophila*, a genome-wide screen identified several redox molecules as essential for the light entrainment of the circadian clock.[68] Similarly, a study in mammals showed that an increase in reduced NADPH and NADH levels enhanced the affinity of the NPAS2 (CLOCK):BMAL1 complex for its target DNA *in vitro*.[69] Thus, redox state may be an important determinant of circadian oscillations in mammalian peripheral tissues.

The circadian clock is thought to have first arisen with the evolution of a eukaryotic lifestyle. This lifestyle requires that the fragile DNA exposed during mitosis be protected from photo-oxidative stress.[70,71] The development of a circadian rhythm would be one way to separate diurnal and nocturnal metabolic processes, with light-dark cycles acting as the selective force. In this scenario, photo-oxidative stress could have been a decisive factor in relegating the anabolic processes of mitosis, growth and consolidation to the dark hours. Thus, it is reasonable to propose that redox signaling is utilized in the control of the circadian clock, and that common regulatory pathways may mediate both cellular responses to photo-oxidative stress and the light entrainment of the circadian clock.

LIGHT-DEPENDENT CIRCADIAN ENTRAINMENT AND DDR SHARE A COMMON SIGNALING PATHWAY

In many organisms, external stimuli are connected to a cell's nucleus *via* MAPK signaling pathways, and roles for MAPKs in circadian clock regulation are well-established.[6,50] There are three major MAPKs: c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK).[72] Light-induced ROS production in zebrafish cells leads to downstream activation of MAPK cascades, which then contribute to the regulation of *zCry1a* transcription[52,57] (Fig. 2). Interestingly, light-induced ERK activation triggers *zCry1a* transcription, whereas light-induced p38 activation suppresses it,[52] highlighting a MAPK-mediated cross-regulatory mechanism of circadian regulation (Fig. 3). We have also found that light directly activates the JNK signaling cascade in zebrafish cells (Uchida *et al.*, unpublished data), an event whose physiological function is under investigation.

Our work has recently shown that light-induced activation of MAPK cascades can contribute to a non-circadian biological process, namely photoreactivation.[52] Photoreactivation is a mechanism by which visible light reverses some of the lethal and mutagenic effects of UV irradiation.[73] Photoreactivation is mediated by DNA photolyases (PHRs), which are DNA repair enzymes.[74] When cells are irradiated with UV light, photoproducts called cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts [(6-4) photoproducts] are produced in the DNA. The PHRs bind to and repair these types of DNA damage using visible light

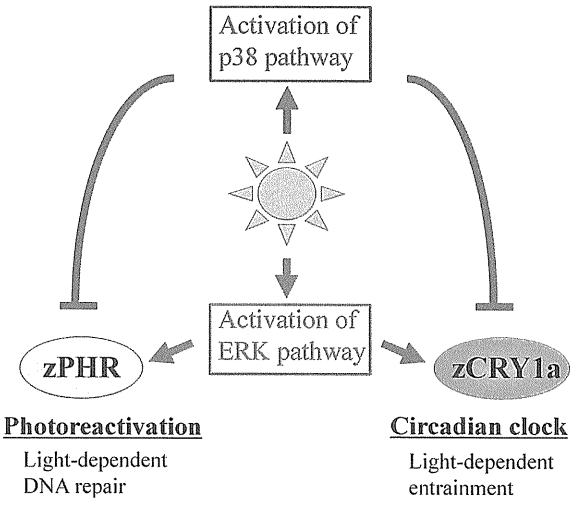

Fig. 3. A Model Depicting the Molecular Mechanisms Underlying Light-Dependent Transcriptional Events Regulating DNA Repair and Circadian Entrainment

Light activates both ERK and p38 MAPK signaling cascades. The former positively regulates expression of both *zPhr* and *zCry1a* genes whereas the latter inhibits it. The light-induced zPHR repairs DNA lesions by utilizing visible light energy. On the other hand, the light-induced zCRY1a entrains the circadian clock by acting as a transcriptional repressor.

as an energy source. Two classes of PHRs have been identified, one specific for CPDs (CPD PHRs) and the other specific for (6-4) photoproducts (64PHRs).[74,75] Both CPD PHRs and 64PHRs are induced by visible light in cultured fish cells.[52,59,76] We have obtained evidence indicating that photoreactivation in zebrafish is a cell-autonomous phenomenon, and that both the induction of 64PHRs in response to light and the subsequent light-dependent repair of DNA by 64PHRs are essential for successful photoreactivation.[52] Notably, the expression levels of the *z64Phr* gene associated with photoreactivation, as well as the *zCry1a* gene associated with the circadian clock, are regulated by the same light-induced MAPK cascades (Fig. 3). Light-induced ERK activation triggers the expression of *z64Phr*, whereas light-induced p38 activation inhibits it.[52] Thus, light-dependent DNA repair and the entrainment of the circadian clock are governed by shared regulatory pathways.

Both CRYs and PHRs belong to the DNA photolyase/cryptochrome protein family and are highly similar in amino acid sequence.[75,77,78] Evolutionary studies have shown that the animal CRY protein first functionally diverged from the CPD photolyase, and then diverged further to generate 64PHR.[79] These facts, together with the observation that *zCry1a* and *z64Phr* share regulatory pathways,[52] strongly indicate an evolutionary link between the circadian clock and DDR. Although solar light has many beneficial uses, including photosynthesis and the entrainment of circadian clock, the UV component of solar energy is harmful to living cells because it produces cytotoxic, mutagenic and carcinogenic lesions in DNA. It is speculated that natural selective pressure must then have forced the development of a self-defense system such as the DNA repair mechanism mediated by DNA PHRs.[75,78] Thus, it is not surprising that two ostensibly very different biological events, repair of UV-damaged DNA and light entrainment of the circadian clock, are governed by the same signaling pathways.

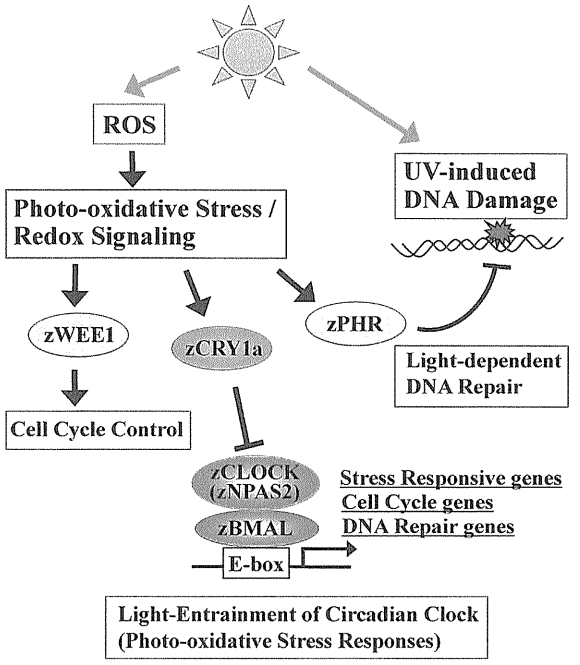

Fig. 4. A Model of Light-Induced Signaling Cascades Potentially Involved in Shared Control of the Circadian Clock, DNA Repair and the Cell Cycle

Sunlight has two major toxic effects: generation of ROS that mediate photo-oxidative stress and induction of UV-mediated DNA damage. In zebrafish, photo-oxidative stress can act as a redox signal that induces expression of a DNA repair gene (*Phr*), a circadian clock gene (*Cry1a*), and a cell cycle regulator gene *(Wee1)*. In the presence of solar light, PHR repairs UV-induced DNA damage and WEE1 halts the cell cycle, protecting the genome of the organism. At the same time, ROS induced by the sun trigger expression of zCRY1a. This transcriptional repressor interacts directly with the zCLOCK (zNPAS2):zBMAL complex and inhibits its transcriptional capacity, thereby entraining the circadian clock. Notably, the zCLOCK (zNPAS2):zBMAL complex also regulates the transcription of a variety of genes involved in cellular stress responses. Thus, it is conceivable that light-dependent circadian entrainment may have originated as a cellular stress response against photo-oxidative stress and/or UV-induced DNA damage.

A COMMON LIGHT-INDUCED SIGNALING PATHWAY REGULATES THE CIRCADIAN CLOCK AND THE CELL CYCLE

Both the cell cycle and the circadian clock are endogenous pacemakers. These mechanisms coexist in most eukaryotic cells and share a number of conceptual features. In particular, both rely on interconnected autoregulatory loops that consist of sequential phases of transcription–translation, protein modification, and degradation.[80] Increasing evidence points to functional links between the cell cycle and circadian rhythms in a variety of organisms.[5,80] In zebrafish, the cell cycle is directly regulated by light. Light determines the timing of S phase entry, establishing a circadian rhythm for cell cycle progression.[58] At the molecular level, light induces the expression of zebrafish *Wee1* (*zWee1*), a cell cycle gene.[60] The Wee1 kinase controls the timing of the G_2-M transition by directly phosphorylating and thus inhibiting Cdc2/cyclin B, leading to suppression of mitotic cell division.[81] This mechanism is consistent with the observation that the growth of cultured zebrafish cells is suppressed by light.[60] Because solar light increases intracellular ROS levels that can exert oxidative stress,[57,65] light-induced *zWee1* expression may act as a cellular stress response, suppressing cell growth under conditions where DNA damage is likely (Fig. 4).

The transcription factor AP-1 modulates a wide range of cellular processes, including cell proliferation, apoptosis and the circadian clock.[82] In mammals, various stimuli activate AP-1, which binds directly to the consensus AP-1 motif within the *Wee1* promoter and drives *Wee1* expression.[83] In zebrafish, light can induce AP-1 activation, which then triggers *zWee1* transcription.[60] Components of AP-1 that are acutely light-inducible include *c-fos*, *fos-B* and *jun-B*.[84,85] In mammals, light-dependent activation of AP-1 in the SCN has been implicated in light-induced phase-shifting of the circadian clock.[84,85] Another factor that induces AP-1 activation is alteration of a cell's redox state. ROS-triggered induction of AP-1 is mediated by MAPK signaling pathways.[86] It is therefore conceivable that a light-induced alteration of cellular redox status could stimulate AP-1 to initiate light-dependent *zCry1a* expression. Indeed, light-stimulated AP-1 activity contributes to *zCry1a* transactivation involved in light entrainment of the circadian clock.[60] Taken together, these findings provide strong evidence that the cell cycle and circadian clock are regulated by a common signaling pathway controlled by AP-1 transcription factor.

PERSPECTIVE

Much evidence has accumulated indicating that evolutionary links exist that have resulted in the functional coupling of the circadian clock and DDR. In *Neurospora*, PRD-4, an orthologue of mammalian Chk2, transduces DNA damage signals into the core circadian machinery, resetting the clock.[87] In the diatom *Phaeodactylum tricornutum*, PtCPF1 (Phaeodactylum tricornutum cryptochrome/photolyase family 1) is a novel cryptochrome/photolyase family member that not only repairs UV-induced DNA damage but also acts as a transcriptional repressor of the circadian clock.[88] In addition, the critical role of redox signaling in the light-dependent entrainment of the circadian clock[57,68] strongly implicates cellular responses to the toxic effects of sunlight as the evolutionary origin of circadian rhythms.

The UV component of sunlight and the photo-oxidative stress derived from it are two major sources of harm to cells (Fig. 4). In lower vertebrates such as zebrafish, the light-induced PHRs repair UV-damaged DNA using light as an energy source.[52] Importantly, this light-induced activation of *DNA Phr* expression appears to be stimulated by photo-oxidative stress.[76] These observations are consistent with the idea that photo-oxidative stress may be utilized as a signal to activate DNA repair enzymes that can protect the organism's DNA from UV-induced damage. The fact that light-dependent expression of *zCry1a* is induced by alteration of a cell's redox state,[57] together with the finding that *zCry1a* and *DNA Phr* are governed by shared light-induced signaling pathways,[52] strongly suggests that oxidative stress may act as a signal triggering the light-induced expression of *zCry1a*. Indeed, H_2O_2, a well-known inducer of oxidative stress, can activate *zCry1a* transcription in zebrafish peripheral cells.[57] Once translated, the zCRY1a protein interacts directly with the CLOCK (NPAS2):BMAL complex and regulates its transcriptional capacity, entraining the circadian clock.[63] The circadian machinery regulates a variety of key genes involved in cellular stress responses, DNA repair, and cell cycle regulation.[24] Thus, zCRY1a may be the key integrator of external signals (such as oxidative stress) that controls the core circadian machinery and regulates the transcription of genes responsible for stress responses, DDR and cell cycle adjustments. It is tempting to speculate that, at least in zebrafish, the light entrainment of the circadian clock reflects a long-standing cellular response to photo-oxidative stress.

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JB Review

Diverse physiological functions of MKK4 and MKK7 during early embryogenesis

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Mitogen-activated protein kinase kinases (MAPKKs) are important components of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signalling pathway. Two MAPKKs that are crucial transducers upstream of JNK signalling are MKK4 and MKK7. These two MAPKKs directly phosphorylate specific Tyr and Thr residues located in the activation loop of the JNK protein and activate this kinase in response to environmental stress, pro-inflammatory cytokines or developmental cues. Although much is known about the biochemical and structural bases of the catalytic mechanism of the MAPKKs, the regulation and physiological functions of these enzymes during early embryogenesis have remained a mystery until relatively recently. Studies employing a range of animal models have now revealed the essential roles that MAPKKs play in diverse developmental contexts, including in dorsoventral patterning, convergent extension and somitogenesis. Focusing primarily on extensive work done in mouse and zebrafish models, this review summarizes the functional properties of MKK4 and MKK7 during vertebrate and invertebrate development, and the mechanisms by which these kinases regulate multiple steps in the establishment of the body plan of an organism.

Keywords: Body plan/early embryogenesis/JNK signalling/MKK4/MKK7.

Abbreviation: CE, convergent extension; Dpp, Decapentaplegic; JIP, JNK-interacting protein; JLP, JNK associated leucine-zipper protein; JNK, c-Jun N-terminal kinase; MKK4, Mitogen-activated protein kinase kinase 4; MKK7, Mitogen-activated protein kinase kinase 7; MO, morpholino; POSH, plenty of Src homology 3; SAPK, stress-activated protein kinase.

Mitogen-activated protein kinase kinase (MKK) 4 and MKK7 are the only molecules known to directly activate the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). Both MKK4 and MKK7 are activated in response to a variety of cellular stresses, including UV and γ-irradiation, heat shock, hyperosmolarity, T cell receptor stimulation, peroxide and inflammatory cytokines. Interestingly, these stress-related enzymes are also activated by developmental cues. In mammals, the JNK family consists of three related genes, *Jnk1*, *Jnk2* and *Jnk3*, which encode 10 protein isoforms. These JNK enzymes phosphorylate a number of transcription factors, including c-Jun, ATF-2, Elk-1, p53 and c-Myc, as well as other proteins such as Bcl-2, Bcl-xL, paxillin and MAP2 (*1–4*). Thus, MKK4 and MKK7 are critical upstream activators of JNK signalling required for developmental programmes and responses to various extracellular stimuli. This review will present the state of our current knowledge on the physiological roles of MKK4 and MKK7 during early embryogenesis in widely divergent species, focusing on the biochemistry and signalling functions of these enzymes in mice and zebrafish.

Biochemical Characteristics of MKK4 and MKK7

MKK4 was first cloned in screens for novel members of the MAPKK family in *Xenopus laevis*, and thus termed XMEK2 (*5*). Subsequently, the homologues of this enzyme were cloned in mouse and human and termed MKK4 (also called SEK1 or JNKK1) (*6–8*). Murine MKK4 is a 397 amino acid protein that contains in its catalytic domain the 11 subdomains found in other protein kinases (Fig. 1A).

Mammalian MKK7 (also called SEK2 or JNKK2) was first identified in the mouse in 1997 (*9–11*). Murine *Mkk7* is most similar to the *Drosophila* JNK activator Hemipterous and mammalian MKK4, sharing 70% and 55% amino acid identity, respectively, within the kinase domain. Mouse *Mkk7* contains 14 exons that can be alternatively spliced to generate a group of protein kinases with three different NH_2-termini (the α-, β- and γ-isoforms) and two different COOH-termini (the 1 and 2 isoforms) (Fig. 1B) (*12*). Comparison of the activities of MKK7 isoforms towards JNK have demonstrated that MKK7α, which lacks the NH_2-terminal extension, exhibits a lower basal activity than the MKK7β- and γ-isoforms (*12*). The physiological relevance of the different MKK7 isoforms remains unclear.

The activities of MKK4 and MKK7 are increased following phosphorylation at Ser and Thr residues within a Ser-X-Ala-Lys-Thr motif in their activation loops. This phosphorylation is mediated by various MAPKKKs, including mixed lineage protein kinases

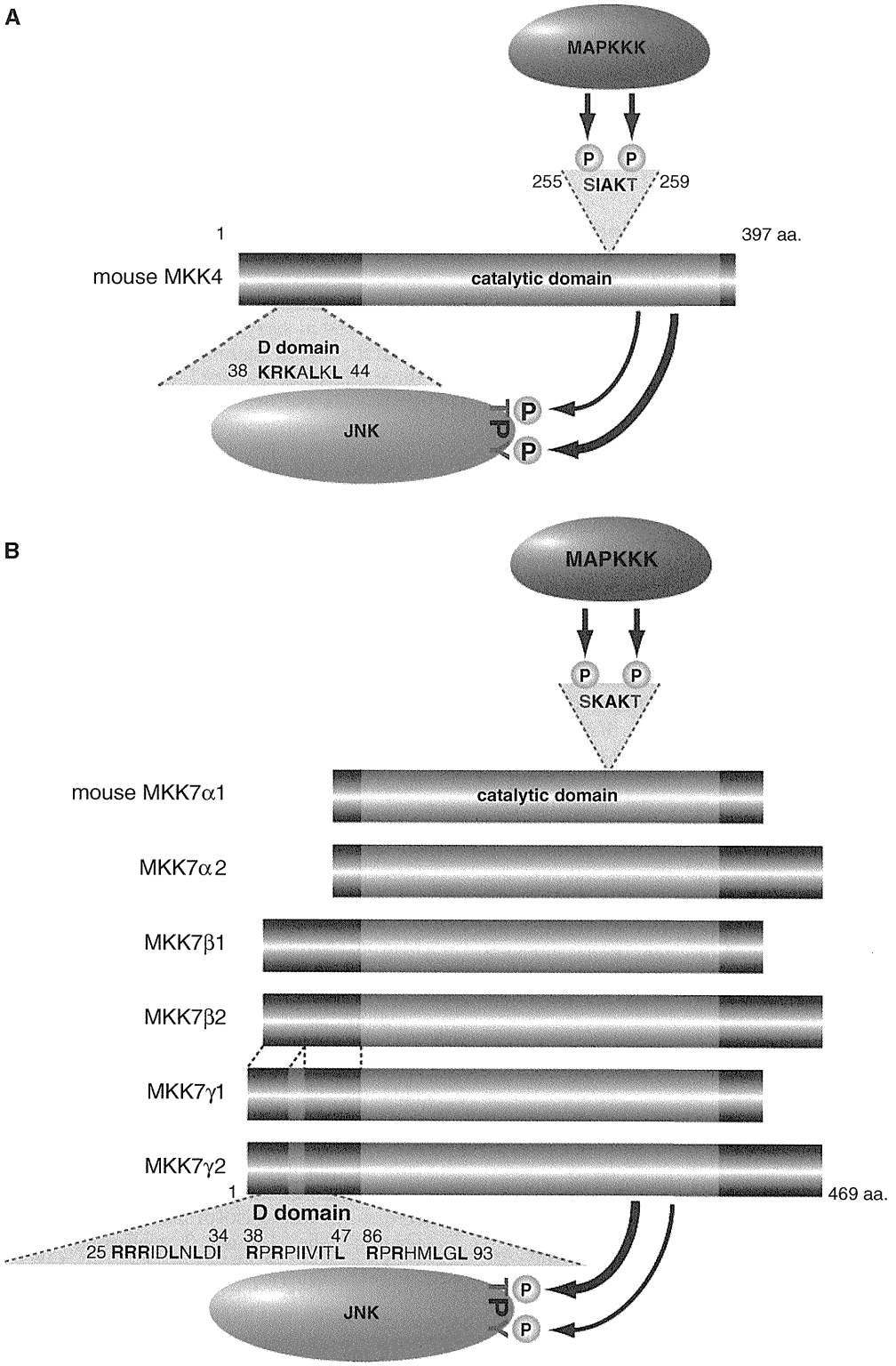

Fig. 1 Function and structure of murine MKK4 and MKK7 isoforms. A specific MAPKKK phosphorylates (A) MKK4 and (B) MKK7 at Ser (S) and Thr (T) residues within the Ser-X-Ala-Lys-Thr (SXAKT) motif of the catalytic domain. Activated dual specificity kinases, MKK4 and MKK7, in turn activate JNK by preferentially phosphorylating the Tyr and Thr residues within a Thr-Pro-Tyr (TPY) motif in JNK's activation loop, respectively (bold arrows indicate preferential phosphorylation). In (B), alternative splicing leads to the inclusion or exclusion of exons located in the 5′- and 3′-regions of the murine *Mkk7* gene, resulting in the generation of the indicated six different MKK7 isoforms that differ in their NH_2- and COOH-termini. The D domain is a JNK docking site in MAPKKs that permits the stable formation of a JNK signalling complex. Residues in the D domains of MKK4 and MKK7 that match the established consensus sequence (*19*, *20*) are depicted in bold.

(MLKs) and MAPK/ERK kinase (MEK) kinase (MEKK1) (*2*, *3*). Activated MKK4 and MKK7 in turn activate JNK by dual phosphorylation of the Thr-Pro-Tyr motif located in JNK's activation loop (Fig. 1A and B). Although MKK4 and MKK7 are dual specificity kinases (Thr and Tyr kinases), previous studies of JNK activation have shown that MKK4 preferentially phosphorylates the Tyr residue, whereas MKK7 phosphorylates the Thr residue. *In vitro* studies have confirmed that phosphorylation of these Tyr and Thr residues results in synergistic activation of JNK (*13–15*). Strong *in vivo* support for this activation mechanism has emerged from studies in our laboratory of mouse embryonic stem (ES) cells bearing targeted disruptions of the *Mkk4* and/or *Mkk7* genes (*16*, *17*). Biochemical analyses of JNK signalling in living ES cells from these animals have demonstrated that Tyr-phosphorylation by MKK4, followed by Thr-phosphorylation by MKK7, leads to synergistic JNK activation in response to stress (*18*).

Scaffold Proteins that Confer Specificity to MKK4 and MKK7 Activities

Multiple mechanisms exist to ensure specificity and prevent cross-talk between components of the MAPK signalling cascade. The specificity of signal transduction by JNK is mediated, in part, by the formation of distinct JNK signalling complexes. These complexes result from interactions between JNK and particular docking sites present on JNK-interacting proteins. The best characterized of these docking sites is the D domain present in MAPKKs. The D domains of MKK4 and MKK7 consist of a cluster of two to three basic residues, followed by a short spacer of 1–2 residues, and a hydrophobic-X-hydrophobic motif (Fig. 1A and B) (*19*, *20*). These MKK docking sites are evolutionarily conserved, and serve to regulate the specificity and enhance the strength of JNK pathway signal transduction. JNK also interacts with various scaffold proteins that can assemble functional signalling modules involving a MAPKKK, a MAPKK and a MAPK (Fig. 2A) (*21*). These scaffold proteins bind specifically to different JNK isoforms and different MAPK and MAPKKs, linking these kinases into a multienzyme complex that provides an insulated physical conduit for signal transduction. Using this conduit, signalling emanating from a particular MAPKK can be transmitted to the appropriate spatiotemporal cellular loci. In this way, MKK4 and MKK7 are responsible for distinct biological functions *in vivo* despite their similarities in sequence and *in vitro* activity.

Several scaffold proteins involved in mammalian JNK signalling modules have been identified, including JNK-interacting protein (JIP) 1, JIP2, JNK/SAPK associated protein 1 (JSAP1)/JIP3, JNK associated leucine-zipper protein (JLP) and plenty of Src homology 3 (POSH) and their various splice variants (*22–29*). JIP1, JIP2 and JSAP1 bind to JNK, MKK7 and various MLKs; JSAP1 associates with JNK, MKK4 and MEKK1; and JLP links Max with

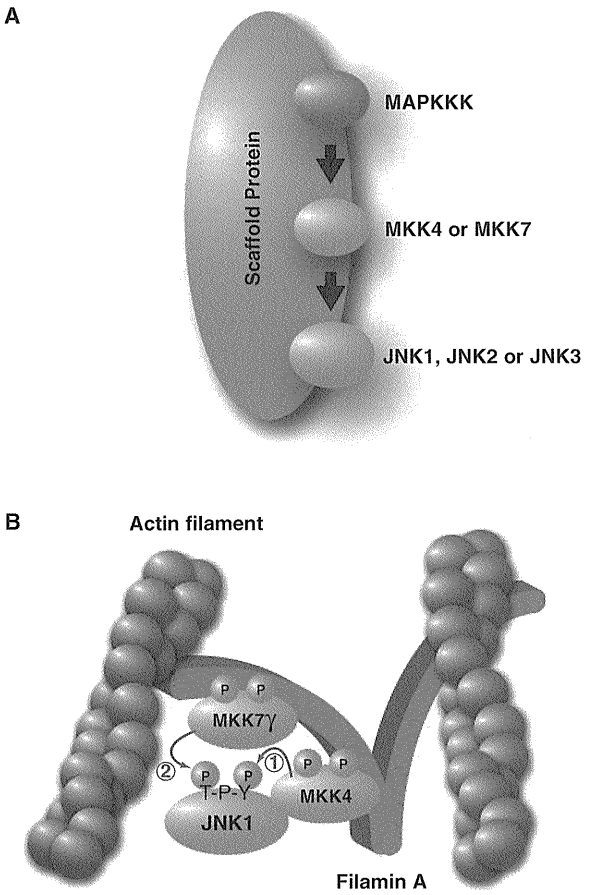

Fig. 2 Scaffold proteins mediating the structural and functional organization of the three-tier JNK signalling module. (A) Model of how a typical scaffold protein supports the assembly of a three-tier JNK signalling module consisting of a MAPKKK, a MAPKK (MKK4 or MKK7) and a JNK. Such scaffold proteins may play a catalytic role as well as an anchoring role depending on the nature of the scaffold protein and the cellular context. (B) Model of how Filamin A acts as a scaffold protein supporting the sequential phosphorylation of JNK by MKK4 and MKK7γ. Filamin A is routinely associated with actin filaments comprising the cytoskeleton. Filamin A also has distinct binding sites for MKK4 and MKK7γ, and can interact simultaneously with both MAPKKs. The interaction of all three proteins with JNK1 leads to synergistic activation of JNK1 in a sequential manner, in which activated (phosphorylated) MKK4 mediates the phosphorylation of the Tyr residue of the Thr-Pro-Tyr motif of JNK (Step 1), followed by Thr-phosphorylation of the same JNK molecule by activated (phosphorylated) MKK7 (Step 2). Filamin A may be the prototype of a novel type of scaffold protein whose function is to link two MAPKKs together and promote synergistic activation of JNK.

c-Myc, and JNK with p38, MKK4 or MEKK3. In addition, multiple upstream MAPKKKs can act as scaffold proteins as well as exert their intrinsic kinase activities. For example, MEKK1 binds to and regulates MKK4. Despite this flexibility, theoretical considerations have dictated that a single JIP-based MAPK module containing MKK4 and MKK7 physically cannot catalyse the sequential phosphorylation of JNK by these kinases. Furthermore, scaffold proteins, such as JIP1, JIP2 and JSAP1, can form homo- and hetero-oligomers (*23*, *24*). Therefore, these scaffolds

could connect two distinct sets of signalling modules, one containing MKK4 and the other containing MKK7. Recently, we identified Filamin A, which interacts with MKK4 (*30*), as a predicted 'binder' protein that can also interact with MKK7 (*31*). Filamin A binds to an NH_2-terminal region present in the MKK7γ and MKK7β splice isoforms but cannot bind to MKK7α, which lacks these amino acids. Experiments using Filamin A deletion mutants revealed that MKK7γ (but not MKK7α) can form a complex with Filamin A and MKK4. This work established a novel model in which MKK4 and MKK7γ utilize Filamin A as a scaffold protein to support their sequential Tyr- and Thr-phosphorylation of JNK and thus its synergistic activation (Fig. 2B) (*31*).

Roles of MKK4 and MKK7 During Early Embryogenesis in Various Species

Phylogenetic analyses of *Mkk4* and *Mkk7* genes have revealed some interesting relationships among species (Fig. 3). Mammals, avians and amphibians appear to have only one gene encoding the MKK4 protein, and these genes are closely clustered in terms of evolutionary distance. In contrast, teleosts such as zebrafish, medakafish and fugu have two Mkk4 genes, *Mkk4a* and *Mkk4b*, and the teleost *Mkk4b* genes are more closely related to each other than to their *Mkk4a* counterparts. These phylogenetic relationships suggest that the duplication of the *Mkk4* gene occurred in the common ancestors of teleosts and tetrapods. With respect to MKK7, all *Mkk7* genes examined to date form a group that includes not only the single *Mkk7* genes from mammalian, avian, amphibian and teleost species but also the one *Mkk7* gene of *Drosophila* and the two *Mkk7* paralogues of nematoda. Neither MKK4 nor MKK7 has been identified in yeast.

Invertebrates

Caenorhabditis elegans. In the nematode *Caenorhabditis elegans*, two homologues of mammalian *Mkk7* have been cloned and are named *mek-1* and *jkk-1* (*32*, *33*). Animals with *mek-1* mutations are hypersensitive to heavy metals and starvation (*33*), and *jkk-1* disruption alters the coordination of body

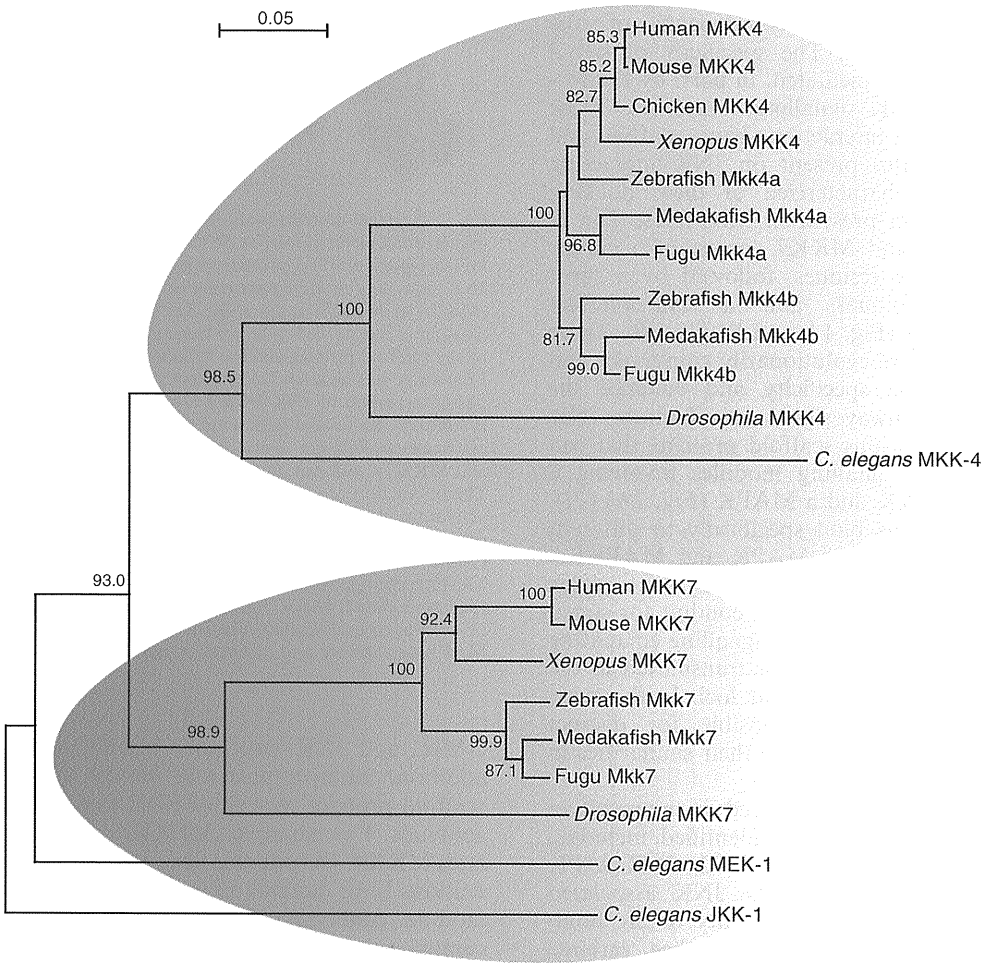

Fig. 3 Molecular phylogenetic tree relating the MKK4 and MKK7 proteins of nine species. The tree was constructed using the neighbour-joining method on the basis of the amino acid identity. The estimated bootstrap probabilities (percent) of local topologies are shown on each node. The length of the scale bar corresponds to an evolutionary distance of 0.05 amino acid substitution per site.

movement via type-D GABAergic motor neurons (*32*). However, neither mutant shows obvious developmental defects. The *C. elegans* genome also contains *mkk-4*, which is highly homologous to mammalian *Mkk4*. The inactivation of *mkk-4* caused an egg-laying defect in hermaphrodites, although there is to date no genetic evidence for MKK4 signalling through JNK in *C. elegans* (*34*). These studies suggest that *mek-1*, *jkk-1* and *mkk-4* are not essential for early embryogenesis in *C. elegans*, but that these genes are important for the regulation of stress responses, locomotion and egg laying.

Drosophila. Genetic studies in *Drosophila* have demonstrated that the JNK pathway is required for early embryonic development in this organism. dJNK (Basket) is activated by dJNKK (Hemipterous), a homologue of vertebrate MKK7 (*35–37*). Basket and Hemipterous are important for morphogenetic processes that involve epithelial cell sheet movement. In the absence of function of either Basket or Hemipterous, lateral epithelial cells fail to stretch and the embryo develops a hole in the dorsal cuticle. The involvement of the JNK pathway in *Drosophila* embryogenesis is further highlighted by the observation that mutants lacking *Drosophila* Jun (dJun) fail to complete dorsal closure (*38–40*). Detailed studies of the process of dorsal closure have demonstrated that dJNK activation is required for dJun phosphorylation and expression of the TGF-β homologue Decapentaplegic (Dpp) in the leading edge of the dorsal epidermis. dFos is also required for Dpp expression (*41*, *42*), indicating that dJNK may trigger Dpp expression by activating an AP-1 complex composed of dFos–dJun heterodimers. Dpp then acts as a secreted signal to control the elongation of lateral epidermis in a paracrine fashion (*43*). These data clearly indicate that the MKK7-JNK signalling pathway has essential functions during early morphogenesis in *Drosophila*.

Although a *Drosophila* orthologue of *Mkk4* has been isolated (*44*), it cannot substitute for Hemipterous (MKK7) function during fly embryonic development because *hemipterous* mutants are embryonic lethal (*35*). Recent genetic and biochemical studies have shown that dMKK4 is dispensable for normal fly development, but that this kinase plays a non-redundant role as a MAPKK acting in parallel to Hemipterous in dTAK1-mediated dJNK activation triggered by Eiger and Imd pathway activation (*45*).

Vertebrates

Mouse. Analyses of various knockout mice have demonstrated the importance of MKK4, MKK7 and JNK signalling in mammalian embryogenesis. $Mkk4^{-/-}$ and $Mkk7^{-/-}$ mice die on embryonic day 10.5 (E10.5) and E11.5, respectively, with severely disorganized livers and reduced hepatoblast numbers (*46–51*). $Jnk1^{-/-}$ $Jnk2^{-/-}$ double mutant mice die at about E11 with defective neural tube morphogenesis and reduced apoptosis in the lateral edges of the hindbrain (*52*, *53*). In contrast, increased apoptosis and caspase activation were found in the forebrain of these double mutants. Thus, the JNK pathway has both pro- and anti-apoptotic effects on the developing mammalian brain.

To determine whether JNK activation is required for the earliest embryonic stages when the vertebrate body plan is first laid down, we recently investigated the effect of combined disruption of the murine *Mkk4* and *Mkk7* genes. $Mkk4^{-/-}$ $Mkk7^{-/-}$ double mutant mice die at about E9.5 (Fig. 4). We examined the progeny of $Mkk4^{+/-}$ $Mkk7^{+/-}$ intercrosses at various developmental stages and found that the expected Mendelian ratio of $Mkk4^{-/-}$ $Mkk7^{-/-}$ embryos (1 : 16) was present at E8.5 but not beyond this point. Intriguingly, $Mkk4^{-/-}$ $Mkk7^{+/+}$ and $Mkk4^{-/-}$ $Mkk7^{+/-}$ mice died earlier than $Mkk4^{+/+}$ $Mkk7^{-/-}$ and $Mkk4^{+/-}$ $Mkk7^{-/-}$ mice, respectively. In addition,

	Genotype									
Mkk4	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-	total
Mkk7	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-	
E8.5	6	19	10	18	47	17	7	23	10*	157
E9.5	7	10	5	12	21	9	5	11 (2)	3 (2)	83
E10.5	4	6	4	6	10	4 (1)	4 (1)	6 (2)	1 (1)	45

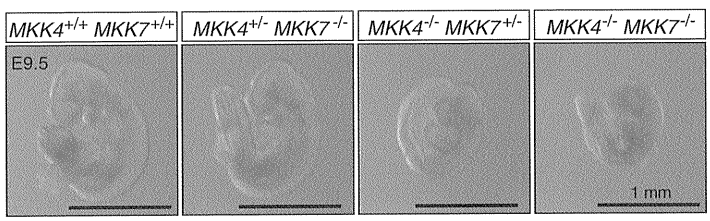

Fig. 4 Embryonic lethality in progeny of $Mkk4^{+/-}$ $Mkk7^{+/-}$ intercrosses. $Mkk4^{+/-}$ $Mkk7^{+/-}$ mice were intercrossed and the genotypes and viability of the progeny embryos were determined at the indicated time points of gestation. Dead embryos (numbers in parentheses) were defined as those in which the heart had stopped beating, as assessed by inverted microscopy. Asterisk indicates severely growth retarded and dying embryos.