Embryonic Growth–Associated Protein Is One Subunit of a Novel N-Terminal Acetyltransferase Complex Essential for Embryonic Vascular Development

Janet M. Wenzlau, Pamela J. Garl, Peter Simpson, Kurt R. Stenmark, James West, Kristin B. Artinger, Raphael A. Nemenoff, Mary C.M. Weiser-Evans

Abstract—N-terminal protein acetylation, catalyzed by N-terminal acetyltransferases (NATs) recognizing distinct N-terminal sequences, is gaining recognition as an essential regulator of normal cell function, but little is known of its role in vertebrate development. We previously cloned a novel gene, embryonic growth–associated protein (EGAP), the expression of which is associated with rapid vascular smooth muscle cell proliferation during development. We show herein EGAP is the mammalian/zebrafish homologue of yeast Mak10p, one subunit of the yeast NatC complex, and describe the cloning of its binding partners Mak3 and Mak31. The EGAP NAT forms a functional complex in mammalian cells, is evolutionarily conserved, and developmentally regulated. It is widely but not ubiquitously expressed during early zebrafish development but undetectable in later developmental stages. We demonstrate EGAP- and Mak3-deficient zebrafish fail to develop because of, in part, decreased cell proliferation, increased apoptosis, and poor blood vessel formation contributing to embryonic lethality. We examined the role of target of rapamycin (TOR), a highly conserved protein kinase controlling cell growth, as a physiological target of EGAP NAT acetylation. Compared with controls, TOR expression and signaling is significantly reduced in EGAP morphants. Pharmacological inhibition of TOR with rapamycin phenocopied the EGAP morpholino oligonucleotide–induced growth and vessel defects. Overexpression of constitutively active TOR rescued EGAP morphants, suggesting TOR is a direct or indirect endogenous substrate of the EGAP NAT complex. These data suggest the EGAP NAT complex is an essential regulatory enzyme controlling the function of a subset of proteins required for embryonic growth control and vessel development. (Circ Res. 2006;98:846-855.)

Key Words: EGAP ■ TOR ■ N-terminal acetyltransferase ■ vascular development ■ vascular cell proliferation

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mooth muscle cell (SMC) proliferation plays a prominent role in normal vessel development and in vascular pathologies, including atherosclerosis and restenosis after angioplasty and stent placement.1–3 During vessel development, SMCs undergo a transient phase of rapid proliferation during which the vessel wall acquires its complement of SMCs.4–8 Replication decreases with vessel maturation as SMCs reach a highly quiescent, differentiated state in adult vessels. However, a hallmark of vascular occlusive disease and interventions in response to disease is a transient increase in SMC replication to levels similar to those exhibited during embryonic life.9,10 SMCs cultured from embryonic vessels or from the neointima of injured vessels during periods of rapid in vivo growth exhibit a unique growth phenotype characterized by rapid serum-stimulated growth and the ability to replicate in a mitogen-independent manner.4,10 This is distinct from SMCs derived from adult uninjured vessels, which exhibit slower serum-stimulated growth and become quiescent under mitogen-deprived conditions. However, the mechanisms regulating this unique SMC growth phenotype at these times have yet to be fully elucidated.

To facilitate our characterization of this rapid proliferative phenotype, we previously cloned several novel genes selectively expressed by rapidly proliferating cells during early vascular development and only re-expressed in the adult vasculature by cells responding to pathological injury.11 One of these genes, which we named embryonic growth–associated protein (EGAP), is expressed in a variety of tissues in the developing rat embryo but restricted in expression in the adult vasculature and is detectable only in tissues undergoing continual cell renewal or in cells responding to pathological injury. We report EGAP, the vertebrate orthologue of the yeast Mak10p protein, is one subunit of a novel N-terminal acetyltransferase (NAT) that is highly conserved among vertebrate species.

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N-terminal acetylation is a post-translational modification of proteins in which the acetyl group of AcetylCoA is transferred to the initiator methionine or most N-terminal amino acid.\textsuperscript{12–14} This irreversible protein modification is catalyzed by a family of NATs, each recognizing distinct N-termini sequences of specific protein substrates. Most information regarding N-acetylation has been obtained from studies in yeast, which express at least three NATs: NatA, NatB, and NatC.\textsuperscript{12,14,15} Each complex consists of independently transcribed subunits; loss or mutation of any one subunit results in loss of NAT activity. N-acetylation may alter protein functions such as protein stability, protein–protein interactions, further modifications such as phosphorylation, and translocation to specific cellular compartments.\textsuperscript{12} For instance, yeast NatB acetylation of actin and tropomyosin is essential for interactions of these proteins and actin filament stabilization.\textsuperscript{16–17} Although many proteins are N-terminal acetylated, there is only a small body of knowledge regarding the role of this protein modification in vertebrate systems. Existing information focuses on the role of the vertebrate Ard1–NatH complex, the yeast NatA-related complex that has the largest number of endogenous substrates identified to date.\textsuperscript{18–21} In contrast, much less is known of the NatC complex, consisting of Mak3p, Mak10p, and Mak31p, or its endogenous substrates.\textsuperscript{22} Subunit copurification and similar slow growth phenotypes of all three yeast deletion strains (mak3p, mak10p, and mak31p) highly suggest that interaction of subunits is required for NatC enzyme activity. However, virtually nothing is known of the biological significance of the EGAP/NatC complex in vertebrate species. Our data establish the EGAP NAT complex functionally coassembles in mammalian cells to regulate cell proliferation and is essential for embryonic development, at least in part through the regulation of target of rapamycin (TOR) signaling events.

**Materials and Methods**

An expanded Materials and Methods section can be found in the online supplement, available at http://circres.ahajournals.org.

**Genomic Sequence Analysis and Morpholino Design**

A positive mouse bacterial artificial chromosome (BAC) clone corresponding to rat EGAP cDNA sequence was obtained from Research Genetics (mouse RPCI BAC 465-E7) and mapped to deduce the genomic structure and complete cDNA sequence. Full-length Flag-, Myc-, and influenza hemagglutinin (HA)-tagged expression constructs were generated for mouse Mak31, EGAP, and Mak3, respectively, by RT-PCR using embryonic day 14 mouse poly(A+) RNA. The genomic organization of the *zebrafish* EGAP and Mak3 loci were deduced from comparisons of their cDNA sequences with genomic trace sequences using the BLAST program on the NCBI website. Antisense morpholino oligonucleotides (MÖs) were designed against the splice donor at the end of the first coding exons.

**In Situ Hybridization, Immunohistochemistry, and TUNEL Staining**

Whole-mount in situ hybridization was performed as described previously. Whole-mount immunohistochemistry was performed using the Vectastain Avidin/Biotin/Horseradish peroxidase ABC Elite System as described previously. TUNEL was used to assess apoptosis in control and EGAP morphants. Briefly, embryos were permeabilized, washed, incubated in terminal transferase (Roche), and biotin-labeled ddUTP (Roche) and biotin incorporation detected with the peroxidase ABC kit (Vector) using diamobenzidine as chromogen. For 5-bromodeoxyuridine (BrdUrd) immunohistochemistry, embryos were incubated with BrdUrd, and 5-μm cryosections were immunohistochemically stained for BrdUrd as described previously.\textsuperscript{4}

**Results**

**Identification and Functional Role of the EGAP NAT Complex**

Verification that EGAP mRNA is developmentally regulated in rat aortic SMCs and sequence analysis showing the EGAP NAT complex is highly conserved can be found in supplemental Figures 1 and 2, available online at http://circres.ahajournals.org.

Studies from yeast proposed NatC subunit expression and assembly are required for its acetyltransferase activity.\textsuperscript{22} Thus, we used coexpression and coinmunoprecipitation analyses to determine whether the mammalian complex similarly colocalizes and functionally assembles in vivo. Human embryonic kidney 293 (HEK 293) cells were transiently transfected with Myc-tagged mouse EGAP (mEGAP), HA-tagged mMak3, and Flag-tagged mMak31 expression constructs to determine cellular localization and to determine overlap in cellular distribution (Figure 1A). All proteins appear in the cytoplasm, are excluded from the nucleus, and show a high degree of overlap in cellular distribution (Figure 1A, merge). To determine whether the mammalian proteins coassemble in living cells, whole cell lysates from transfected HEK 293 cells were immunoprecipitated using anti-Myc or anti-Flag antibodies, and Western blotting was performed using anti-Myc, anti-Flag, or anti-HA antibodies (Figure 1B). Myc-mEGAP, HA-mMak3, and Flag-mMak31 were detected in cells overexpressing all three subunits using either anti-Myc or anti-Flag for immunoprecipitation (Figure 1B, lanes 1 and 8), demonstrating mEGAP, mMak3, and mMak31 coassemble in mammalian cells. The results from these reciprocal immunoprecipitation experiments strongly suggest formation of a stable in vivo complex in mammalian cells. Omission of mMak31 (Figure 1B, lanes 3 and 9), mMak3 (Figure 1B, lanes 4 and 10), or mEGAP (Figure 1B, lanes 5 and 11) from transfections resulted in loss of subunit assembly, especially recruitment and association of mMak3, the catalytic subunit, with mEGAP and mMak31 (mEGAP and mMak31 continued to associate in the absence of mMak3; Figure 1B, lanes 4 and 10).

Because the mammalian EGAP NAT proteins form a stable in vivo complex and EGAP is associated with rapid cell proliferation,\textsuperscript{13} we determined the functional role of subunit assembly on cell growth. Transfected HEK 293 cells were incubated in low serum media in the presence of BrdUrd for 24 hours, and the degree of BrdUrd incorporation, and therefore DNA synthesis, was determined. Compared with nontransfected cells that begin to exhibit a decline in replication after 24 hours in low serum, cells overexpressing all three subunits maintained high rates of BrdUrd incorporation (Figure 1C). Omission of mEGAP, mMak3, or mMak31 from transfections resulted in reduced levels of BrdUrd incorporation (similar to nontransfected cells), highly suggestive of a
functional role for the EGAP NAT complex in regulating cell proliferation.

The EGAP NAT Complex Is Essential for Zebrafish Development

Our previous data suggested expression and subsequent downregulation of EGAP plays an important role in development and differentiation, including vascular cell growth and differentiation. We next used the zebrafish model to determine the biological significance of the EGAP NAT during development. Using whole-mount in situ hybridization, we found zEGAP mRNA is expressed at high levels in the developing zebrafish embryo as early as 6 hours post fertilization (hpf) and remains high in many developing tissues through 18 hpf and 28 hpf (Figure 2Aa through 2Ac). Consistent with our previous studies in the rat, zEGAP expression was high in developing vascular structures, implicating an important role in blood vessel development (Figure 2Ad through 2Ag). Staining was also strong in the epidermis but not the neural tube or notochord (Figure 2Ag). Staining becomes restricted to the developing head by 48 hpf (Figure 2Ah) and by 5 days post fertilization (dpf), is observed only in the epidermis (Figure 2Ai), where actively replicating cells were detected (Figure 2Aj). The temporal and spatial pattern of zMak3 expression is very similar to that of zEGAP (Figure 2B), and both are similar to those we reported previously for EGAP in the developing rat; thus, EGAP and Mak3 are evolutionarily conserved, developmentally regulated genes.

To establish the EGAP NAT is essential for zebrafish development, we diminished EGAP expression using MOs targeted to the splice donor site at the end of EGAP exon 2 (containing the ATG start codon). Microinjection of 6 ng MO
into zebrafish embryos produced a truncated form of EGAP through insertion of a partial intron sequence (Figure 3A). Compared with control MO-injected embryos, EGAP morphants exhibited significant delays in growth (Figure 3B and 3C), and very few live EGAP morphants were observed after 4 to 5 dpf. At the 14-somite stage (16 hpf), 88% (150 of 170) morphants presented with 12 somites compared with controls, and chevron/V-shaped somites normally observed beginning at this stage, a measure of developmental stage, were not observed in the morphants (Figure 3B). Therefore, 16-hpf morphants were developmentally equivalent to 14.5-hpf controls. By 24 hpf, morphants were noticeably smaller, and many structures exhibited a developmental delay compared with controls (91% [111 of 122] of 6-ng EGAP morphants presented with <12 somites compared with controls, and chevron/V-shaped somites normally observed beginning at this stage, a measure of developmental stage, were not observed in the morphants (Figure 3B). Therefore, 16-hpf morphants were developmentally equivalent to 14.5-hpf controls. By 24 hpf, morphants were noticeably smaller, and many structures exhibited a developmental delay compared with controls (91% [111 of 122] of 6-ng EGAP morphants presented with a similar phenotype; Figure 3C). Body length of morphants, a quantitative measure of developmental age after the 14-somite stage, was significantly decreased at 24 hpf compared with controls (Figure 3C). The majority of morphants exhibited pericardial and hindbrain edema by 48 hpf (Figure 3C), and by 4 to 5 dpf, severe pericardial edema was accompanied by apparent heart failure, likely causing lethality. To determine an effect on cell proliferation, controls and EGAP morphants were incubated with BrdUrd, fixed at 18 hpf, and BrdUrd incorporation determined by immunohistochemistry. Whereas controls showed extensive and ubiquitous cell proliferation at this stage, few BrdUrd-positive cells were present in morphants (Figure 3D). In contrast, significantly elevated numbers of apoptotic cells were detected in morphants (Figure 3E), indicating an essential role for EGAP in embryonic cell proliferation and survival. The EGAP MO-induced phenotype and lethality was rescued by overexpression of human cytomegalovirus immediate-early enhancer/promoter (CMV)-EGAP, verifying the observed phenotype was specific to EGAP depletion (Figure 3F). MO mediated depletion of zMak3 phenocopies the EGAP MO-induced phenotype (Figure 3B and 3C); combined with similar expression patterns and subunit assembly, these data are consistent with EGAP and Mak3 functioning together.

Because zEGAP and zMak3 mRNA levels were high in developing vascular structures (Figure 2), we used fli1-enhanced green fluorescent protein (EGFP) transgenic zebrafish to determine the role of the EGAP NAT in vessel development. Expression of fli1, an Ets domain family member, in the developing zebrafish begins early and is predominantly mesodermal but becomes restricted to differentiating angioblasts, endothelial cells, primitive blood cells, and pharyngeal arch mesenchyme, therefore serving as an early marker for endothelial cells.23–25 Control or EGAP-specific MO-injected Fli1-EGFP transgenic zebrafish were analyzed for EGFP expression at 15 and 26 hpf (Figure 4). EGFP fluorescence was detected throughout the anterior and posterior lateral mesoderm at 15 hpf in controls (Figure 4Ab). In contrast, morphants exhibited reduced EGFP fluorescence at 15 hpf (Figure 4Ac through 4Af), consistent with reduced cell proliferation at this time. Morphants exhibiting a phenotype (eg, reduced somite number; Figure 4Ac through 4Ae) expressed less EGFP compared with those that did not exhibit...
an obvious phenotype (Figure 4Af). At 26 hpf, EGFP expression in controls was similar to endogenous fli1 expression (Figure 4Ba through 4Bd). Patent dorsal aortae and posterior cardinal veins, with visible circulating blood cells, and well-formed segmental vessels were apparent in controls. In contrast, EGAP morphants exhibited less developed, disorganized vessels at 26 hpf (Figure 4Be through 4Bg). Major trunk vessels failed to form adequate lumens, and compared with controls, segmental vessels were truncated (Figure 4B, graph). Circulating blood cells present were often observed accumulated in proximal main trunk vessels, and little segmental vessel perfusion was observed even at later stages (data not shown). The morphant vessel phenotype was rescued by overexpression of CMV-EGAP (Figure 4C), implicating an essential role for the EGAP NAT in proper vessel development, likely through the regulation of vascular cell proliferation. Because EGAP is not restricted to the vasculature and cardiac failure was observed in morphants, we analyzed the developing heart to determine whether vessel defects are secondary to depressed cardiac development or function. We detected no obvious differences in heart rhythm at 24 and 48 hpf; both controls and morphants exhibited strong and coordinated contractility (data not shown) and little differences in heart rate (supplemental Figure 3A). ncx1h (sodium calcium exchanger) and cmcl2 (cardiac myosin light chain), two cardiac-specific differentiation markers, were expressed in normal temporal and spatial patterns in both controls and morphants (supplemental Figure 3B). These data suggest heart development and function are occurring normally, and heart failure occurs in response to primary defects in other tissues, including the vasculature.

**Mechanism of Action of the EGAP NAT**

A family of NATs catalyzes N-terminal acetylation of eukaryotic proteins, and each NAT recognizes distinct
N-terminal sequences. In yeast, subclasses of proteins with Met-Ile, Met-Leu, Met-Trp, or Met-Phe N-termini are acetylated by yeast NatC/EGAP; NatC has the most limited number of identified protein substrates. To begin to define its mechanism of action, we focused on the mammalian TOR (mTOR) as a potential target of the EGAP NAT. TOR, a large serine/threonine protein kinase lying downstream of growth factor receptor signaling, is integral in coordinating cell growth and proliferation. Inactivation of Drosophila TOR results in reduced cell size and embryonic lethality. Similarly, mTOR null mouse embryos die shortly after implantation because of impaired cell proliferation, demonstrating mTOR is essential for embryonic development. mTOR activity is essential for vascular cell proliferation during vessel formation, and our previous data demonstrated rapid growth of embryonic SMCs is dependent on constitutive phosphatidylinositol 3-kinase–mTOR signaling events. Further, the TOR N termini is Met-Leu, providing the intriguing possibility that TOR is a direct EGAP NAT substrate. To determine whether the EGAP NAT regulates TOR function, control and EGAP morphants were analyzed at 24 hpf by whole-mount immunohistochemistry for total and phosphorylated zTOR (zTOR) levels. We found high and ubiquitous expression of total and phosphorylated zTOR in controls (Figure 5A). In contrast, both total and phosphorylated zTOR were barely detectable in EGAP morphants (Figure 5A). Immunohistochemical data were verified by Western analysis of pooled controls or EGAP morphants (Figure 5B). In addition, phosphorylation of p70S6 kinase on T389, a site directly mediated by mTOR, was significantly reduced in EGAP morphants compared with controls (Figure 5B), strongly suggesting the proliferative defect in EGAP-deficient zebrafish embryos is, at least in part, mediated through decreased expression or signaling of zTOR.

To determine whether decreased TOR plays a role in mediating EGAP MO-induced growth defects, wild-type and fli-EGFP transgenic zebrafish embryos were incubated with the TOR inhibitor rapamycin. Compared with vehicle controls, rapamycin phenocopied EGAP morphant body length and vessel defects (Figure 6A). Low dose (10 nmol/L) resulted in similar defects as observed in EGAP morphants, whereas a higher dose (100 nmol/L) generated more severe defects, suggesting loss of TOR signaling is central to the observed EGAP-deficient phenotype. To confirm that loss of TOR signaling is involved in the EGAP MO-induced phenotype, we coinjected embryos with EGAP MO and constitutively active CMV-mTOR. Compared with EGAP MO injection alone, overexpression of mTOR in coinjected embryos rescued somite, body length, and vessel defects and lethality observed in EGAP morphants (Figure 6B). These data provide novel evidence that EGAP NAT activity is an essential regulator of TOR activity and, consequently, embryonic development.

Discussion

Rapid growth and proliferation of embryonic cells is essential for development of all organ systems and is responsible for...
postnatal/pathological injury repair processes. Regulation occurs through the coordinated expression and activity of a host of transcription factors, ligands and receptors, and intracellular signaling molecules. Therefore, it is necessary to understand both the mechanisms controlling gene expression and the post-translational modifications regulating the function of proteins involved in these processes. Through our studies to identify novel genes for which expression is high in the developing rat vasculature, absent in adult vessels, but increased in the setting of vascular injury, we cloned the vertebrate orthologues of the yeast NatC complex. Although many proteins are N-acetylated, there is only a small body of knowledge regarding the role of this protein modification in vertebrate systems, and virtually nothing is known of the functional role of the EGAP NAT during development. We show here that EGAP and Mak3 are expressed in overlapping temporal and spatial patterns during zebrafish development and that all three mammalian subunits specifically interact to form a stable, functional complex in vivo. To our knowledge, this is the first report showing the consequences of a lack of N-acetylation on vertebrate developmental processes. In particular, we are the first to show loss of EGAP/NatC-induced acetylation results in embryonic lethality marked, at least in part, by reduced cell proliferation and survival and attenuated vessel development, indicating EGAP NAT-induced acetylation is essential for embryonic development. In addition, we identified TOR as a downstream target of EGAP NAT activity. Reduced cell proliferation combined with increased cell death observed in EGAP morphants is consistent with cell growth and survival functions of the TOR signaling pathway, thus strongly implicating disruption of TOR signaling events as central to the proliferative defect in EGAP-deficient zebrafish embryos.

We first identified EGAP in rapidly proliferating vascular SMCs for which re-expression after vascular injury is associated with pathological neointima formation. In addition, another group showed EGAP expression correlates with cornea and skin maturation and re-epithelialization of wounded cornea, suggesting a role for EGAP NAT acetylation in epithelial differentiation, and wound healing. EGAP/Mak10p was first identified as a glucose-repressible gene necessary for replication of a dsRNA virus of Saccharomyces cerevisiae. Mak10p- and Mak3p-deficient yeast mutants fail to assemble the L-A dsRNA viral particle because of decreased acetylation of the killer viral major coat protein, Gag, and exhibit similar slow growth phenotypes on nonfermentable carbon sources. Only a few yeast NatC substrates have been identified, including the GTPase, Arl3p, and the mitochondrial proteins Kdg1p and Fum1p. Acetylation is necessary for recruitment to Golgi membranes for membrane trafficking (Arl3p) and for growth on nonfermentable carbon sources (Kdg1p and Fum1p). Our data expand on the role of the vertebrate complex, demonstrating it is essential for cell growth and survival and proper embryonic development at least in part through the regulation of TOR signaling events. Furthermore, functional subunit interactions and dynamic regulation of EGAP and Mak3 indicate control of this system occurs.

Figure 5. EGAP-deficient zebrafish embryos exhibit reduced total and phosphorylated zTOR levels (A). Twenty-four-hpf controls and EGAP morphants were immunohistochemically stained using anti-total mTOR (Aa and Ac) and anti-phosphoS2448mTOR (Ab and Ad) antibodies. Positive reaction color is brown. Aa and Ab, Controls; Ac and Ad, zEGAP morphants. Arrowheads, head. Yolk sacs were removed for visualization. Embryos are viewed anterior to the left (B). Embryos described above were collected at 16 or 24 hpf, pooled, and whole cell lysates collected for Western analysis. Western blots were probed (IB) using anti-phosphoS2448-mTOR, anti-total mTOR, and anti-phosphoT389 S6 kinase antibodies. Shown at left are positions of molecular weight markers.
through the regulation of enzyme activity and EGAP NAT subunit expression. EGAP is expressed in several, but not all, tissues in the developing zebrafish, and EGAP NAT activity is likely essential for proliferative events associated with development of these tissues. Our data suggest a model whereby loss of EGAP NAT function causes primary defects in the developing vasculature and other affected tissues (eg, epidermis), resulting in a developmental delay, pericardial edema, and cardiac failure. We cannot definitively rule out the possibility that vascular abnormalities observed in the morphants are secondary events. However, because morphant hearts develop two contracting chambers, express differentiated markers in the correct pattern, and vessel defects are observed before heart abnormalities, it is likely that morphants exhibit primary vessel defects rather than secondary to cardiac dysfunction.

The EGAP/NatC complex and TOR proteins are evolutionarily conserved. Both are found in yeast, plants, worms, flies, and higher vertebrate species, and TOR is a principal regulator of embryonic development by controlling cell growth. Reduced TOR expression and signaling in EGAP-deficient zebrafish embryos combined with a similar phenotype after pharmacological inhibition of TOR and a rescued phenotype by TOR overexpression is com-

Figure 6. Rapamycin phenocopies and constitutively active mTOR cDNA rescues EGAP MO-induced defects (A). Wild-type (left) or Fl1-EGFP transgenic (right) zebrafish embryos were incubated with rapamycin from the 8-cell-stage until 26 hpf (right) or 28 hpf (left). Aa, Vehicle control; Ab, zEGAP morphant; Ac, 10 nmol/L rapamycin; Ad, 100 nmol/L rapamycin (B). Constitutively active mTOR-rescued wild-type (left) and Fl1-EGFP transgenic (right) embryos. Ba, Control; Bb, zEGAP morphant; Bc, zEGAP MO and CMV–mTOR coinjection. Y indicates yolk sac. Body lengths and segmental vessel lengths were measured as described in Figures 3 and 4. Data are presented as the means±SD; *different from control MO; **different from zEGAP MO; P<0.001. All embryos are lateral view, anterior to the left.
pelling evidence that TOR is an important downstream target of EGAP NAT activity. Whether TOR is a direct EGAP NAT substrate has yet to be determined. The second amino acid residue of mTOR is leucine, an EGAP/NatC consensus sequence, suggesting this is a distinct possibility. Preliminary data in our laboratory show that EGAP NAT-rich systems express an acetylated form of TOR, whereas TOR acetylation is not observed in EGAP-deficient systems (data not shown). Alternatively, it is possible that TOR is not a direct EGAP NAT substrate but rather a downstream effector of an unidentified EGAP NAT substrate(s). Nevertheless, we were able to drive an almost complete rescue of the EGAP MO-induced phenotype with injection of constitutively active mTOR cDNA placing the TOR signaling pathway as a major target of this acetyltransferase. Our current studies are designed to determine whether TOR is a direct endogenous substrate and define the functional consequences of decreased TOR acetylation in EGAP NAT–deficient systems, which would add to the short list of proteins for which function is dependent on EGAP/NatC-induced acetylation and to define additional endogenous EGAP NAT substrates.

Recent findings indicate N-terminal acetylation is a highly conserved essential regulator of many biological processes, including embryonic growth control, vessel development, and SMC function. Distinct NAT mutant phenotypes, substrate specificity, and dynamically regulated subunit expression patterns indicate N-terminal acetylation of select protein substrates is a nonredundant, highly regulated controller of protein function. The available evidence suggests the EGAP NAT provides a novel regulatory role on a small subset of proteins, including TOR, essential for embryonic cell proliferation and growth. Identification of additional endogenous substrates and its role in pathophysiological processes will lend further insight into the biological significance of this acetyltransferase in higher vertebrate species and could serve as a fundamental platform for the design of a new class of pharmaceuticals useful in inhibiting a panel of proteins involved in pathological proliferative events.

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SUPPLEMENTAL INFORMATION

MATERIALS AND METHODS

Animals and Cells
Zebrafish were maintained, embryos raised, and staged according to hours post-fertilization (hpf) at 28.5°C and morphological criteria as described\(^1\). Fli1-GFP transgenic (TG(fli1:nEGFP)y7) zebrafish, expressing nuclear localized EGFP under the control of the fli1 promoter\(^2\), were obtained from the Zebrafish International Resource Center (University of Oregon, Eugene, OR). Embryonic day 17 (Emb) and adult (Ad) rat aortic SMC were subcultured as described previously\(^3\). Human embryonic kidney 293 (HEK293) cells were maintained in high glucose DMEM containing 10% FCS and used for transfections, immunofluorescence, and immunoprecipitation assays. All experiments involving vertebrate animals were approved by the University of Colorado Health Sciences Center Animal Care and Use Committee and performed in accordance with relevant guidelines and regulations.

Reagents
Monoclonal anti-Myc (clone 9E10), anti-Flag (clone M2), and anti-Flag-cy3 (clone M2-cy3) and bromodeoxyuridine (BrdU) were from Sigma. Monoclonal anti-HA (clone 12CA5) was from Roche. Monoclonal anti-BrdU was from Becton Dickinson. Polyclonal anti-total mTOR, anti-phospho\(^{52448}\)mTOR, and anti-phospho\(^{T389}\)S6 kinase were from Cell Signaling. Alexa488-conjugated anti-mouse antibody and a monoclonal antibody Alexa 546 (cy3) labeling kit were from Molecular probes. \(^3\)H-AcetylCoA (1.85 MBq) and Amplify fluorography reagent were from Amersham. Morpholino-modified oligonucleotides (MOs) were purchased from Gene Tools. Rapamycin was from Biomol Research Laboratories.
Genomic sequence analysis, plasmids, morpholino design, and injections

A positive mouse BAC clone corresponding to rat EGAP cDNA sequence was obtained from Research Genetics (mouse RPCI BAC #465-E7), subcloned into pBS+ and mapped to deduce the genomic structure and complete cDNA sequence (putative transcription start site determined by 5’RACE; Genbank accession numbers: AY102701 and AY102702). cDNA clones representing human and zebrafish EGAP were identified in the GenBank database by searching for sequences homologous to mouse EGAP (accession numbers NM024635 [human] and NM199550 [zebrafish]). cDNA clones representing mouse, human, and zebrafish Mak3 and Mak31 were identified in the GenBank database by searching for sequences homologous to yeast Mak3p and Mak31p (accession numbers for Mak3: BC0557117 [mouse], NM025146 [human], BC078316 [zebrafish]; accession numbers for Mak31: NM030083 [mouse], AA09944 [human], AW077254/fj33f12.y1 [zebrafish, EST from Trace Archives]). Full-length Flag-, Myc-, and HA-tagged expression constructs were generated for mouse Mak31, EGAP, and Mak3, respectively, by RT-PCR using embryonic day 14 mouse poly(A+) RNA. cDNAs were subcloned into pCMV-Tag1 (Mak31), pCMV-Tag3 (EGAP), or pcDNA-Script (Mak3; vectors from Stratagene); an HA tag was added to the 5’ end of Mak3. A CMV promoter-based plasmid containing full-length constitutively active rat mTOR was a kind gift from Dr. Gary Chiang (The Burnham Institute) and was described previously. 300-bp EGAP and Mak3 cDNA fragments were generated by RT-PCR using wild-type 24 hpf zebrafish embryo poly(A+) RNA; cDNAs were subcloned into pcRII (Invitrogen) and used to produce RNA probes for whole mount in situ hybridizations. Plasmids encoding zebrafish cardiac myosin light chain (cmcl2) and the zebrafish sodium calcium exchanger, ncxlh, two cardiac-specific markers, were generously provided by Dr. Deborah Garrity (Colorado State University) and were used to generate RNA
probes for whole mount in situ hybridizations. The genomic organization of the zebrafish EGAP and Mak3 loci were deduced from comparisons of their cDNA sequences with genomic trace sequences using the BLAST program on the NCBI website. Antisense morpholinos (MO) were designed against the splice donor at the end of the first coding exons. EGAP: 5’-TGTAAGGTGTGTGTCTGTGT-3’; Mak3: 5’-TGAAAGGTTAGTAGACGACAGAGA-3’. Control MO had the sequence 5’-CCTCTTACCTCAGTTACAATTTATA-3’. MOs were dissolved in distilled water and 3-6 ng and 10 kDa lysinated Fluoresein dextran (LFD; Molecular Probes) were injected into the yolk of 1- to 8-cell-stage embryos; total volume of injections was 10 nl. For rescue experiments, 100 pg CMV-EGAP or CMV-active mTOR was first injected into the cell of 1-cell-stage embryos with LFD (total volume 3-6 nl) followed by 6 ng EGAP MO between 1- and 4-cell stage. Body length and segmental vessel length were measured using the MetaMorph image acquisition, processing, and analysis system. Body length was determined by measuring a minimum of 25 embryos per condition. Segmental vessel length was determined by measuring >3 midtrunk segmental vessels per embryo and a minimum of 25 embryos per condition.

**Semi-quantitative RT-PCR**

Total RNA was extracted from Emb or Ad SMC or from dechorionated 16 hpf zebrafish embryos using Tri-reagent (Sigma), DNase-treated to remove contaminating DNA, and 2 μg RNA was reverse transcribed using random hexamers and Superscript reverse transcriptase (Invitrogen). For semi-quantitative PCR, 2 μl (EGAP) or 1 μl (β-actin) cDNA template was amplified using Platinum PCR supermix (Invitrogen) in 50 μl reaction volumes; 10 μl aliquots were removed at cycle numbers 23, 26, and 29. Gene-specific primers are as follows: EGAP: 5’-AATGGTTATGAAAGCCGCAGTTAGG-3’ and 5’-CCTTGAGCCATGTGATCAAACA-3’;
β-actin: 5’CATCCGTAAAGACCTC TATGCCAAC-3’ and 5’-CAAAGAAAGGGTGTAAAACGCAGC-3’.
PCR for zebrafish EGAP was performed for 30 cycles using the following primers: 5’-ATGGTG ATGAAGTCATCGGTCGA-3’ and 5’-TGGCGGACATGGCTTCGAACAGA-3’.

Transfections
HEK293 cells were plated on 60 mm cell culture plates the day before transfection. One µg each CMV-Myc-EGAP, CMV-HA-Mak3, and CMV-Flag-Mak31 were transfected into the cells using Effectene transfection reagent (Qiagen) as described previously. Culture media were changed after 18 h and cells recovered for an additional 24 h. For some samples, equimolar amounts of an empty CMV plasmid were substituted for Myc-EGAP, HA-Mak3, and/or Flag-Mak31.

Immunofluorescence, immunoprecipitation, and immunoblotting
Cells were fixed in 4% paraformaldehyde, permeabilized in PBS + 0.2% Triton X-100, blocked with 5% horse serum in PBS for 60 min and incubated overnight at 4°C with anti-Myc or anti-HA antibodies followed by Alexa488-conjugated anti-mouse antibodies for 60 min. Cells were then incubated overnight at 4°C with cy3-conjugated anti-Flag or anti-Myc antibodies, mounted for immunofluorescence microscopy using Vectashield with DAPI (Vector Laboratories) to label cell nuclei and examined using a Zeiss LSM410 laser-scanning confocal microscope. For co-immunoprecipitation, transfected HEK293 cells were harvested in RIPA buffer containing 1% Nonidet P-40 and protease inhibitors (10 µg/ml aprotinin and leupeptin, 1 mM PMSF). Lysates were centrifuged and the supernatant pre-cleared by incubating with rabbit IgG and protein A-sepharose on ice for 60 min. 500 µg of precleared lysate was incubated with 1 µg of anti-Myc or anti-Flag antibodies overnight at 4°C followed by 100 µl of protein A slurry for 2 h. Immunoprecipitates were washed 3 times with RIPA buffer and suspended in 20 µl SDS-PAGE
sample buffer. Myc-tagged EGAP, HA-tagged Mak3, and Flag-tagged Mak31 were detected using Western blotting as described previously\textsuperscript{7}. For Western analysis of zebrafish proteins, 16- and 24-hpf zebrafish embryos were harvested and dechorionized, lysed at 4°C in lysis buffer, and equal amounts of protein were subjected to SDS-PAGE (NOVEX system, Invitrogen Corp., Carlsbad, CA). Western analysis was conducted as previously described\textsuperscript{8,9}.

**In situ hybridization, immunohistochemistry, and TUNEL staining**

Whole mount in situ hybridization was performed as described previously\textsuperscript{10}. EGAP, Mak3, *cmcl2*, and *ncx1h* DIG-labeled sense and antisense probes were generated (Roche Diagnostics), embryos were hybridized with DIG-labeled probes and hybridizations detected with alkaline phosphatase-conjugated anti-DIG antibodies. Bound antibody was visualized using NBT/BCIP (Gibco BRL). Whole mount immunohistochemistry was performed using the Vectastain Avidin/Biotin/Horseradish peroxidase ABC Elite System as described previously\textsuperscript{11}. Embryos were fixed in 4% paraformaldehyde, permeabilized with proteinase K, blocked overnight in 1% goat serum, then sequentially incubated with primary antibody (1:50), biotin-labeled secondary antibody, and ABC complex; incubations were all performed overnight at 4°C. Antigen-antibody complexes were detected with DAB. TUNEL (terminal transferase mediated dUTP nick end-labeling) was used to assess apoptosis in control and EGAP morphants. Embryos were fixed at 24-hpf as for in situ hybridization and stored in methanol. After rehydration, embryos were permeabilized with 0.1% sodium citrate, washed in PBT and incubated in terminal transferase buffer (Roche) on ice for 1 hour with terminal transferase (Roche) and biotin-labeled ddUTP (Roche) followed by 1 hour incubation at 37°C. Embryos were extensively washed in PBT and biotin incorporation detected with the peroxidase ABC kit (Vector) using DAB as chromogen. Stained embryos were post-fixed in 4% paraformaldehyde and visualized using an
inverted Zeiss microscope equipped with video imaging. For BrdU immunohistochemistry, MO-injected embryos were incubated with BrdU (10 mM final concentration in fish water) from 4 hpf to 16 hpf, fixed in 4% paraformaldehyde, and embedded in OCT reagent. 5 μm cryosections were immunohistochemically stained for BrdU as previously described\textsuperscript{12} and counterstained with hematoxylin.

**Statistical Analysis**

Data are expressed as the means±SD. Differences among groups were analyzed by one-way ANOVA followed by Fisher’s protected least significant difference using Statview software. Significance was accepted at p<0.05.
RESULTS

Identification and Functional Role of the EGAP NAT Complex

We first identified EGAP in rapidly proliferating SMC whose re-expression after vascular injury is associated with neointima formation\(^\text{14}\). EGAP is highly expressed in embryonic vessels, is virtually undetectable in mature, uninjured vessels, but is re-expressed at high levels in balloon-injured carotid arteries. Using semi-quantitative RT-PCR, we verified EGAP mRNA is expressed at higher levels in proliferating rat Emb SMC maintained under serum-free conditions compared to proliferating rat Ad SMC maintained in 10% CS (Supplemental Figure 1). EGAP mRNA was expressed >2.5-fold higher in Emb SMC in agreement with our previous data\(^\text{14}\); EGAP mRNA was undetectable in growth-arrested serum-deprived Ad SMC (not shown). In addition, EGAP mRNA was expressed at high levels in Emb SMC under both serum-deprived and serum-stimulated conditions, environments where Emb SMC readily proliferate\(^3,8,12\).

Further sequence analysis showed rat EGAP has significant homology to yeast Mak10p, one subunit of the yeast NatC\(^\text{13}\). Mouse, human, zebrafish, c. elegans, and drosophila EGAP homologues were identified in the NCBI database. EGAP is highly conserved among species; rat EGAP shares 99% identity to the mouse and human proteins, and 96% identity to the zebrafish protein at the amino acid level (Supplemental Figure 2A). Yeast NatC consists of three subunits, Mak10p, Mak3p, and Mak31p\(^\text{13}\). Since EGAP is the mammalian homologue of yeast Mak10p, we next identified and cloned mouse Mak3 and Mak31. Similar to EGAP, mouse, human and zebrafish Mak3 and Mak31 orthologues were identified and found highly conserved among species; mouse proteins share 98% (human Mak3), 96% (fish Mak3), 97% (human Mak31), and 82% (fish Mak31; partial analysis) identity at the amino acid level (Supplemental Figure 2B&C).
**FIGURE LEGENDS**

*Supplemental Figure 1. EGAP mRNA is preferentially expressed by embryonic-derived SMC.*

Top: Total RNA was extracted from embryonic day 17 (Emb)-derived rat aortic SMC maintained in SFM for 72 hrs or from adult (Ad)-derived rat aortic SMC maintained in 10% CS. Semi-quantitative RT-PCR was conducted using EGAP-specific primers. Shown in the left panel is a representative PCR. EGAP signals were normalized to β-actin and are presented in the graph as the means±SD. Bottom: Semi-quantitative RT-PCR was conducted on Emb SMC maintained in SFM or 10% CS for 72 hrs. Shown in the left panel is a representative PCR. EGAP signals were normalized to β-actin (graph).

*Supplemental Figure 2. The EGAP NAT complex is highly homologous across species.* The amino acid sequences for mouse, human, zebrafish, and rat (EGAP only) were aligned for (A) EGAP, (B) Mak3, and (C) Mak31. Red residues indicate differences from mouse sequences.

*Supplemental Figure 3. Heart development and function occur normally in EGAP morphants.* (A). Zebrafish embryos were injected at the 2-8-cell stage with 6ng of control- or EGAP-specific MOs or co-injected with EGAP MO plus CMV-EGAP (EGAP rescue) and heart rate (beats per minute) assessed at 24-, 28-, 50-, and 72-hpf. (B). Whole mount in situ hybridizations were performed on 28- and 50-hpf controls (top) or EGAP morphants (bottom) using *cmcl2*- or *ncx1h*-specific DIG-labeled RNA probes (reaction color blue-purple; pink is background staining). Arrowheads = developing hearts.
REFERENCES


Supporting Figure 1
Supporting Figure 2
Supporting Figure 3

A. 
Heart Rate (Beats Per Minute) vs. Hours post-fertilization (hpf)

- Control MO
- EGAP MO
- EGAP rescue

B. 

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<tr>
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<th>cmlc2</th>
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Control MO

EGAP MO