Regulation of cell proliferation by ERK and signal-dependent nuclear translocation of ERK is dependent on Tm5NM1-containing actin filaments

Galina Schevzova,b,*, Anthony J. Keeb,f, Bin Wanga,* Vanessa B. Sequeirab, Jeff Hooka, Jason D. Coombsa,c, Christine A. Luscha, Justine R. Stehnb, Elizabeth A. Musgrovea,b,1, Alexandra Cretusa, Richard Assoina, Thomas Fathc, Tamara Hanoc, Ron Segerd, Irina Pleinesb, Benjamin T. Kil eb, Edna C. Hardemanh,i, and Peter W. Gunninga,b,2

1Oncology Research Unit, School of Medical Sciences, 2Cellular and Genetic Medicine Unit, and 3Neurodegeneration and Repair Laboratory, School of Medical Sciences, University of New South Wales, Australia, Sydney, NSW 2052, Australia; 4Sydney Medical School, University of Sydney, Sydney, Sydney, NSW 2006, Australia; 5Kinghorn Cancer Centre, Cancer Research Program, Garvan Institute of Medical Research, Darlinghurst, Sydney, NSW 2010, Australia; 6Department of Pharmacology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6160; 7Department of Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel; 8Cancer and Hematology Division, Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia

ABSTRACT ERK-regulated cell proliferation requires multiple phosphorylation events catalyzed first by MEK and then by casein kinase 2 (CK2), followed by interaction with importin7 and subsequent nuclear translocation of pERK. We report that genetic manipulation of a core component of the actin filaments of cancer cells, the tropomyosin Tm5NM1, regulates the proliferation of normal cells both in vitro and in vivo. Mouse embryo fibroblasts (MEFs) lacking Tm5NM1, which have reduced proliferative capacity, are insensitive to inhibition of ERK by peptide and small-molecule inhibitors, indicating that ERK is unable to regulate proliferation of these knockout (KO) cells. Treatment of wild-type MEFs with a CK2 inhibitor to block phosphorylation of the nuclear translocation signal in pERK resulted in greatly decreased cell proliferation and a significant reduction in the nuclear translocation of pERK. In contrast, Tm5NM1 KO MEFs, which show reduced nuclear translocation of pERK, were unaffected by inhibition of CK2. This suggested that it is nuclear translocation of CK2-phosphorylated pERK that regulates cell proliferation and this capacity is absent in Tm5NM1 KO cells. Proximity ligation assays confirmed a growth factor–stimulated interaction of pERK with Tm5NM1 and that the interaction of pERK with importin7 is greatly reduced in the Tm5NM1 KO cells.

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E14-10-1453) on May 13, 2015.

*These authors contributed equally.

© 2015 Schevzov, Kee, Wang, et al. This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).

Abbreviations used: CK2, casein kinase 2; ERK1/2, extracellular signal-regulated kinases 1/2; Imp7, importin 7; JNK, c-Jun-N-terminal kinase; KO, knockout; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; NTS, nuclear translocation sequence; P38K, phosphatidylinositol 3-kinase; PLA, proximity ligation assay; qRT-PCR, quantitative real-time-PCR; SPS, Ser-244/Pro-245/Ser-246; Tm, tropomyosin; Tm5NM1, tropomyosin isoform 5 nonmuscle 1; WAT, white adipose tissue; WT, wild type.

Monitoring Editor Valerie Mane Weaver University of California, San Francisco

Received: Oct 16, 2014 Revised: May 5, 2015 Accepted: May 7, 2015
INTRODUCTION
Constitutive activation of the mitogen-activated protein kinase (MAPK) signaling pathways is the primary drive of cell proliferation in most cancers (Robertson and Der, 2007). These pathways consist of distinct tiers of conserved protein serine/threonine kinases that define each pathway and include the well-characterized extracellular signal-regulated kinases 1/2 (ERK1/2, termed here ERK), c-Jun-N-terminal kinase (JNK), p38 MAPK (Roskoski, 2012), and ERK5 (Nithianandarajah-Jones et al., 2012). Activation of each MAPK requires dual phosphorylation of specific residues within the activation loop. For example, ERK is initially phosphorylated by MAPK/ERK kinase (MEK) on the Thr-Glu-Tyr (TEY) motif (Segre and Krebs, 1995), with subsequent phosphorylation on the Ser-244/Pro-245/Ser-246 (SPS) nuclear translocation sequence (NTS) mainly by casein kinase 2 (CK2) to generate pSPS-pERK (Chuderland et al., 2008; Plotnikov et al., 2011). These events lead to the nuclear translocation of ERK by the interaction of the phosphorylated NTS with the nuclear shuttling protein importin7 (Imp7) (Chuderland et al., 2008). This cascade of well-defined events is essential for ERK-induced cell cycle regulation (Weber et al., 1997; Schwartz and Assoian, 2001).

The actin cytoskeleton is a key regulator of numerous cellular functions, including proliferation and entry into the S phase of the cell cycle (Bohmer et al., 1996; Huang et al., 1998; Huang and Engber, 2002; Reshetnikova et al., 2000); however, the mechanism by which the cytoskeleton regulates cell proliferation has not been well defined. There is increasing evidence of interaction between actin filaments and components of the MAPK signal transduction pathways, with early reports suggesting a dependence on actin filaments for MAPK-regulated cell proliferation (Leinweber et al., 1999; Aplin et al., 2001; McNicol et al., 2001; Harrison et al., 2004; Smith et al., 2004; Jongstra-Bilen and Jongstra, 2006; Ren et al., 2007; White et al., 2009). Several reports detected ERK in the Triton X-100-insoluble fraction, which is consistent with an association with the actin cytoskeleton (Atten et al., 1998; Leinweber et al., 1999; Schrick et al., 2007). Morgan and coworkers showed capping of ERK with actin stress fibers and found that release of ERK from stress fibers might be required for activation of ERK (Vetterkind et al., 2013). Thus the actin cytoskeleton has been seen as a cytoplasmic anchor for ERK and potential inhibitor of ERK function.

The weakness with this view of the role of the actin cytoskeleton is the intrinsic assumption that the actin filaments as visualized by phalloidin staining or fractionated by Triton X-100 solubility are compositionally generic. Instead, it has become increasingly clear that this is not true (Michelot and Drubin, 2011). The functional diversity of actin filament populations is spatially segregated and functionally distinct (Schevzov et al., 1992; Dugina et al., 2009; Bunnell and Ervasti, 2011). In addition, tropomyosin (Tm) is an integral component of most actin filaments in all metazoan cells (Gunning et al., 2008). The specific Tm incorporated into actin filaments, at least in yeast, is specified by the formin used to build the actin filament (Johnson et al., 2009; Bunnell and Ervasti, 2011). The functional diversity of actin filament populations is controlled, at least in part, by the Tm component of actin filaments (Gunning et al., 2008; O’Neill et al., 2008). It is therefore possible that if the actin cytoskeleton is involved in cell proliferation, it will be mediated by a specific population of actin filaments containing a specific Tm.

Transformation of cells is accompanied by profound changes in the Tm isoform composition of their actin cytoskeleton (Hendricks and Weintraub, 1984; Lin et al., 1985). There are only two Tm isoforms consistently retained by all human cancer cells thus far examined, tropomyosin isoform 5 nonmuscle 1 (Tm5NM1) and Tm4 (Stehn et al., 2006, 2013). We previously reported that Tm5NM1 is essential for embryonic stem cell proliferation (Hook et al., 2011) and cancer cell survival (Stehn et al., 2013) and that expression of this isoform increases after release of NIH3T3 cells from quiescence back into the cell cycle (Percival et al., 2000; Stehn et al., 2013). These results point to Tm5NM1-containing actin filaments in actin-mediated cell proliferation. We used genetically manipulated mice and cells derived from them to test the role of Tm5NM1 in regulating cell proliferation in vivo and in vitro. Tm5NM1 was found to be required for the regulation of cell proliferation by ERK and the signal-dependent nuclear translocation of ERK.

RESULTS
Tm5NM1 promotes cell proliferation in vitro
To determine whether Tm5NM1 has a role in cell proliferation, we analyzed primary MEFs isolated from wild-type (WT) and Tm5NM1 knockout (KO) and transgenic (TG) mice. Tm5NM1 TG MEFs express twofold higher levels of Tm5NM1 than WT MEFs, whereas Tm5NM1 expression is undetectable in the KO MEFs (Figure 1, A and B). In cell proliferation assays using synchronized cells, we found that TG MEFs had an enhanced cell proliferation, whereas KO MEFs displayed reduced cell proliferation (Figure 1C). The observed defect in cell proliferation was also evident at lower (2.5%) and higher (15%) serum levels (Supplemental Figure S1). We observed a similar reduced proliferation rate in Tm5NM1 KO MEFs harvested from mice of a different genetic background, indicating that the decrease in proliferation is robust (Supplemental Figure S2, A and B). Restoration of Tm5NM1 expression by mating KO and TG mice rescued the phenotypes, and MEFs isolated from these mice have a proliferation rate similar to MEFs harvested from WT mice (Figure 1, D–F). Flow cytometry analysis of the Tm5NM1 KO MEFs showed no significant changes in the percentage of cells detected at different phases of the cell cycle compared with WT MEFs (Supplemental Figure S3A). This is an expected outcome. We predict that the observed proliferation defect is likely due to a change in G1. Owing to the slow doubling time of these cells (36–48 h; unpublished data) together with the observation that ~60% of the cells are in G1 (Supplemental Figure S3A), it is unlikely that a statistically significant change in the fraction of cells in G1 would be detected. In addition, knocking out or overexpressing Tm5NM1 does not result in compensation by other Tm isoforms (Supplemental Figure S3, B–D).

To confirm that the decrease in proliferation was due to an isoform-specific effect, we examined the effect of the loss of Tm4, another low–molecular weight Tm isoform consistently expressed in both normal and cancer cells (Stehn et al., 2013), on cell proliferation. Functional knockout of Tm4 in Tpm4Plt53/Plt53 MEFs had no effect on proliferation relative to WT MEFs (Figure 1, G–I). Of importance, depletion of Tm4 protein had no effect on the levels of Tm5NM1 (Figure 1G). Collectively these data indicate that the effect on proliferation observed in the KO and TG MEFs is specifically a consequence of altered Tm5NM1 expression levels.

It is well established that cell proliferation is influenced by cell–substratum interactions, and the actin cytoskeleton is implicated in this biological response (Assoian and Klein, 2008). In particular, cells display a graded proliferative response to changes in substratum compliance (Klein et al., 2009). In this study, the reduced proliferation rate of Tm5NM1 KO MEFs was unrelated to their ability to respond to changes in substratum compliance (Supplemental Figure S3E).

Tm5NM1 promotes cell proliferation in vivo
To determine whether perturbation of Tm5NM1 levels in vivo has any effect on cell proliferation, we analyzed tissues from Tm5NM1

2476 | G. Schevzov, A. J. Kee, B. Wang, et al. Molecular Biology of the Cell
KO and TG mice described previously (Bryce et al., 2003; Kee et al., 2004; Vlahovich et al., 2009). Most striking was the variation in weight of white adipose tissue (WAT) depots between these mouse lines (Figure 2, A and B). The weight of both abdominal (epididymal and retroperitoneal) and subcutaneous (inguinal) WAT depots as a percentage of body weight was increased in adult (3-mo-old) TG mice and reduced in KO mice compared with WT controls (Figure 2, A and B). Similar changes occurred in brain and kidney mass, with a significant decrease observed in KO mice and an increase in TG mice (Figure 2, C and D). Total body fat mass was higher in the TG mice and reduced in the KO mice (Supplemental Figure S4A). However, the body weights of TG and KO mice were similar to that of their WT controls (Supplemental Figure S4B) since there was a significant decrease and increase in lean body mass in TG and KO mice, respectively, as determined by dual x-ray absorptiometry (Supplemental Figure S4A).

WAT mass was unchanged in a TG mouse overexpressing a different Tm isoform, Tm3 (Schevzov et al., 2008), and in mice expressing a functional null Tm4 protein (Tpm4^{Pit53/Plt53}; Figure 2, E and F). In addition, brain and kidney masses of the Tm3 and Tp^{Pit53/Plt53} mice were similar to those of their respective control mice (Figure 2, G and H). Collectively these data indicate an isoform-specific role for Tm5NM1 in the size of fat pads, brain, and kidney in vivo.

To assess whether the changes in TG and KO fat pad sizes were a consequence of altered proliferation of adipocytes, we measured DNA content. DNA content per whole fat pad was significantly increased in adult WAT from TG mice and decreased in the KO WAT compared with WT WAT (Figure 3, A and B, respectively). These data are consistent with changes in adipocyte cell number accounting for the changes in WAT mass in the Tm5NM1 TG and KO mice. Changes in cell proliferation in vivo were measured using the cell cycle marker Ki67 (Figure 3, C–E). The TG kidney showed a
the effect of Tm5NM1 on tissue and organ weight is consistent with altered cell proliferation in at least WAT and kidney and suggests that the observed effect of Tm5NM1 on MEF proliferation is of physiological significance in the mouse.

ERK regulation of cell proliferation requires Tm5NM1
Cyclin D1 expression plays a significant role in controlling cell cycle progression through the G1 phase, and its expression has been linked to signaling by the ERK subfamily of MAP kinases (Weber et al., 1997). Furthermore, MAPK signaling via ERK and cyclin D1 expression is actin dependent (Bohmer et al., 1996; Welsh et al., 2001). Therefore to determine whether Tm5NM1 is acting through the MEK/ERK/cyclin D pathway, we examined the expression of key members of this pathway in KO and TG MEFs and adipose tissue from TG mice. Consistent with previous studies (Welsh et al., 2001), we observed that addition of serum (24-h time point) to quiescent WT MEFs led to the induction of cyclin D1 expression (Figure 4). In contrast, in the KO MEFs, addition of serum (24 h) failed to increase the level of cyclin D1 expression, whereas a significant increase was observed in the TG MEFs.

The transcription factor Elk-1 is a major and direct nuclear downstream target of MAPK. Activation of the MAPK pathways and in particular ERKs results in the rapid and efficient phosphorylation and activation of Elk-1 (Yoon and Seger, 2006). Analysis of the nuclear localization of pElk-1 conducted in MEFs from both strains of mice (129/Svj and C57BL/6) showed that the KO MEFs displayed a significant reduction in the percentage of the cells with nuclear pElk-1 (Figure 4, E–H) after serum stimulation.

We conducted microarray gene expression profiling on epidydimal adipose tissue of 14-wk-old TG vs. WT mice. The complete list of significantly altered genes (n = 1283) is available at the Gene Expression Omnibus (series record GSE25013). The Gene Ontology categories most significantly overrepresented (enriched) in the differentially regulated genes were Cellular Growth and Proliferation and Cell Cycle (Figure 4I). The category terms Cell Morphology and DNA Transcription were also overrepresented in the TG epidydimal adipose tissue (Figure 4). To verify the changes in cell cycle gene expression detected by the microarray, we performed quantitative real-time PCR (qRT-PCR) on cDNA synthesized from extracts of adult TG and WT control WAT (n = 10 samples/genotype). Both E2F1 and cyclin D2 (the major cyclin D isoform in fat) were significantly enhanced in the TG WAT compared with control (Figure 4J).
proliferation rate compared with vehicle (Figure 5A). The KO MEFs were sensitive to inhibition of MEK, JNK, p38, and PI3K (Figure 5B) but completely resistant to ERK2 inhibition. We treated cells with two known ERK inhibitors, ERK inhibitor peptide 1 (Rasola et al., 2010) (Figure 5B,C) and the ERK11e inhibitor (Aronov et al., 2009; Figure 5, D and E), both shown to inhibit primarily ERK2. Moreover, the resistance of the KO MEFs to ERK inhibitor peptide 1 is independent of serum stimulation, as log phase (asynchronous cultures) responded in a similar manner (Figure 5F). Thus the KO MEFs have lost their ability to respond to ERK stimulation of cell proliferation.

Nuclear translocation of pERK is reduced in Tm5NM1 KO MEFs
The failure of the KO MEFs to be responsive to ERK stimulation of proliferation might be due to dysfunction at several steps in the ERK pathway. The first of these is activation of ERK by phosphorylation of its TEY motif by MEK. In agreement with previous studies (Welsh et al., 2001), we observed activation of ERK after serum stimulation of synchronized MEFs (Figure 6A). The levels of pERK were unchanged in Tm5NM1 TG and KO MEFs relative to WT after serum addition (Figure 6B). No change in the expression of total ERK was detected in either the TG or KO MEFs compared with WT cells (Figure 6A).

Nuclear trafficking of pERK is essential for G1-phase cell cycle progression (Chen et al., 1992; Lenormand et al., 1993; Brunet et al., 1999). To determine whether Tm5NM1 has a functional role in transporting pERK into the nucleus, we performed a single-cell immunofluorescence assay to visualize nuclear pERK. A gradual increase in the number of cells with nucleus-localized pERK was seen in both WT and TG, with no significant differences detected between them during the first 30 min after serum stimulation (Figure 6, C and D). In contrast, KO MEFs had a significantly reduced fraction of cells with nuclear pERK after 10 min of serum stimulation compared with WT MEFs (57 ± 1.11% vs. WT MEFs, 69 ± 0.82%; Figure 6, C and D). Although a change in nuclear pERK was detected between the WT and KO MEFs, we found that this difference was insufficient to be detected by isolation of nuclear and cytoplasmic subfractions followed by Western blotting (unpublished data). Restoration of Tm5NM1 in the KO MEFs rescued the pERK nuclear translocation defect seen in the KO MEFs (Figure 6E). This suggests a defect in the translocation of pERK to the nucleus in cells devoid of Tm5NM1.

Tm5NM1 is required for translocation of SPS-phosphorylated pERK into the nucleus
The defect in nuclear translocation of pERK in the KO cells could be due to a lack of phosphorylation of activated pERK on the
We observed elevated (although not statistically significant) rather than reduced levels of SPS-phosphorylated pERK in the KO cells after serum stimulation and normal levels of CK2 (Figure 7, A and B). This suggests that Tm5NM1 is acting downstream of CK2 phosphorylation of pERK and predicts that the KO cells would be insensitive to inhibition of CK2. WT and KO MEFs were treated with a CK2 inhibitor before serum stimulation. Proliferation of WT MEFs was significantly reduced by CK2 inhibition, whereas the growth of KO MEFs was unaffected compared with their respective vehicle controls (Figure 7, C and D). In addition, there was a 30% reduction in the translocation of pERK to the nucleus in serum-stimulated WT MEFs treated with the CK2 inhibitor (Figure 7E). This indicates that it is primarily the SPS-phosphorylated nuclear ERK that is responsible for ERK-stimulated cell proliferation and is significantly reduced by CK2 inhibition. The fraction of KO MEFs that were positive for nuclear pERK was similar to that of WT MEFs treated with the CK2 inhibitor, and the KO MEFs were

**FIGURE 4:** Evidence for changes in the expression of cyclin D, pElk-1, and E2F with altered Tm5NM1 levels. (A, C) Representative Western blots probed with cyclin D1 and α-tubulin antibodies of the 129/SvJ WT and KO MEFs and the C57BL/6 WT and TG MEFs, serum starved, and then stimulated to proliferate with the addition of serum for 24 and 96 h. (B, D) Quantification based on n = 3 blots using α-tubulin as loading control. *p < 0.05, **p < 0.01. (E, G) WT (wt/wt) and KO (ko/ko) MEFs from the 129/SvJ and C57BL/6 strains of mice, were synchronized by serum starvation, followed by addition of serum for 15 min and immunofluorescence staining with pElk-1 (red). Nuclei were detected with DAPI (blue). Scale bar, 10 μm. (F, H) Percentage of cells with positive pElk-1 nuclear stain measured in the presence of 15 min of serum (>1500 cells/group). ****p < 0.0001. (I) Gene enrichment analysis of Illumina mRNA array data from 14-wk-old Tm5NM1 TG (tg/tg) white adipose tissue (epidydimal fat) indicates that Tm5NM1 affects preferentially cell proliferation and cell cycle genes. Ingenuity Systems Analysis was performed on all transcripts shown by Illumina array analysis to be up-or down-regulated by ≥1.5 (n = 1283) in TG vs. WT fat. (J) Quantitative RT-PCR analysis of selected cell cycle genes of 14-wk-old TG (tg/tg) and WT (wt/wt) epididymal adipose tissue. Shown are the fold changes from WT controls. n = 10/group, *p < 0.05 (ANOVA).
pERK) is responsible for cell proliferation and suggest that it is pSPS-pERK that is not translocated to the nucleus in the absence of Tm5NM1.

refractory to the CK2 inhibitor (Figure 7E). The data indicate that the fraction of nuclear pERK that depends on CK2 phosphorylation (pERK phosphorylated on the SPS site, referred to as pSPS-pERK) is responsible for cell proliferation and suggest that it is pSPS-pERK that is not translocated to the nucleus in the absence of Tm5NM1.

FIGURE 5: Tm5NM1 KO MEFs are refractory to ERK2 inhibition. (A, B) Serum-starved, synchronized WT (wt/wt), Tm5NM1 TG (tg/tg), and KO (ko/ko) MEFs were pretreated for 30 min with vehicle alone (DMSO) or the MEK1/2 (UO126, 2.5 μM), ERK peptide 1 (2.5 μM), JNK (JNK1/2, 1 μM), p38 (SB202190, 10 μM), or PI3K (LY294002, 2.5 μM) inhibitor, replated with serum, and cells counted. Proliferation rate was determined from the slope of the linear regression curves and plotted as a fold change relative to DMSO-treated MEFs. (C, D) Representative proliferation curves for serum-stimulated WT (wt/wt) and Tm5NM1 KO (ko/ko) MEFs in the presence of DMSO or ERK-specific inhibitor determined as described in A and B. (F) Representative proliferation curves for asynchronous WT (wt/wt) and Tm5NM1 KO (ko/ko) MEFs in the presence of DMSO or the ERK-specific peptide inhibitor 1. n = 3, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
FIGURE 6: pERK nuclear translocation is impaired in TmSNM1 KO MEFs. (A) Serum-starved WT (wt/wt), TmSNM1 TG (tg/tg), and KO (ko/ko) MEFs were serum stimulated for 5 and 15 min and cell extracts analyzed by Western blotting using pERK, total ERK and α-tubulin (to evaluate protein loading). Representative blots are shown. (B) Quantitation of pERK. (C) WT (wt/wt), TmSNM1 TG (tg/tg), and KO (ko/ko) MEFs were synchronized by serum starvation, followed by addition of serum for 15 min, and immunofluorescence stained with pERK. Nuclei were detected with DAPI. Arrows indicate nuclei. Scale bar, 10 μm. (D) Percentage of cells with greater nuclear pERK signal relative to cytoplasm measured in the absence, 0 min, and the presence of serum for 3, 5, 10, 15, and 30 min (n = 3). (E) Percentage of cells with greater nuclear pERK signal relative to cytoplasm measured in TmSNM1 TGxKO (tg/ko) MEFs after 15 min of serum addition. **p < 0.01.
Inhibition of WT MEFs with cytochalasin D had a similar effect on nuclear translocation of pERK to that seen in the KO MEFs (Figure 7, F and G). However, nuclear translocation of pERK in the KO MEFs was insensitive to cytochalasin D (Figure 7, F and G). This suggests that the effect of cytochalasin D on pERK nuclear translocation is dependent on Tm5NM1 and mediated by Tm5NM1-containing actin filaments.

**pERK and Imp7 interactions are impaired in the absence of Tm5NM1**

The simplest interpretation of these data is that Tm5NM1-containing actin filaments are required for the interaction of pERK with Imp7. To test this, we used the Duolink in situ proximity ligation assay (PLA) to evaluate the interaction of Tm5NM1 with pERK and the effect of the Tm5NM1 KO on the ability of pERK to interact with Imp7 (Soderberg et al., 2006). pERK interacts with Tm5NM1 in a serum-responsive manner, with a significantly higher number of PLA signal dots in the WT MEFs after serum addition relative to serum-starved cells (Figure 8, A and C). We then examined the interaction of pERK with its nuclear shuttling protein Imp7 (Chuderland et al., 2008). In WT MEFs, serum stimulation resulted in a significant increase in the number of pERK and Imp7 interactions (Figure 8, B and D). In Tm5NM1 KO MEFs, serum stimulation resulted in an increase in pERK and Imp7 interactions; however, the number of interactions was significantly lower than in the WT MEFs (Figure 8, B and D). This is not due to changes in Imp7 levels, as the WT and KO MEFs have similar levels of expression of Imp7 (Figure 8). We also confirmed that in the presence of the ERK2 inhibitor, there is a significant reduction in the number of pERK/Imp7 interactions in both the WT and KO MEFs (Figure 8G). Finally, the decrease in pERK and Imp7 interactions occurs in a Tm isoform-specific manner, as no significant change in the number of interactions between these proteins is seen in the Tm4 KO MEFs after 15-min serum stimulation (Figure 8H). This suggests that Tm5NM1-containing actin filaments are required for the association of pSPS-pERK with Imp7 and subsequent nuclear importation of pSPS-pERK to promote cell proliferation.

**DISCUSSION**

One of the difficulties with identifying the physiological roles of the actin cytoskeleton is that anti-actin drugs cause such widespread disruption of the cell cytoplasm that it becomes difficult to separate primary from secondary effects. In contrast, the total genetic elimination of specific tropomyosin isoforms is a potentially powerful approach to identifying the function of subsets of actin filaments containing just one or two tropomyosins in mammals (Vlahovich et al., 2009; Hook et al., 2011) and yeast (Drees et al., 1995; Skau et al., 2009; Clayton et al., 2010; Coulton et al., 2010). These previous studies detected the effect of tropomyosin isoforms on cell structure and myosin motor function, which is not unexpected, given the fundamental role of actin filaments in these processes. However, this report is the first to identify a role for a tropomyosin at a specific step in a signaling pathway and raises the possibility that other tropomyosin isoforms might regulate distinct steps in signaling pathways.

The MAPK signal cascade plays a vital role in cell proliferation through its ability to regulate cell cycle entry (Chen et al., 1992; Lenormand et al., 1993; Diehl et al., 1998; Brunet et al., 1999), and constitutive activation of the ERK/MAPK pathway has been identified as the driver of uncontrolled cancer cell growth (Roberts and Der, 2007). Previous studies provided evidence that actin filaments are involved in the organization of signal transduction networks, and there is increasing evidence for a direct association between actin filaments and components of the MAPK pathway (Leinweber et al., 1999; Harrison et al., 2004; Jongstra-Bilen and Jongstra, 2006; Ren et al., 2007; White et al., 2009). The specific nature of this interaction has been difficult to interpret, however, due to the use of anti-actin drugs. More recently, Dugina et al. (2015) showed that γ-actin, which usually contains Tpm3.1 in its filaments (Schevozov et al., 1993), promotes tumorigenesis and interacts with activated ERK1/2. This aligns strongly with our results. Our identification of a specific role for Tm5NM1 in the ERK/MAPK pathway provides the opportunity to both dissect the mechanism in molecular detail and also provide an explanation for why tumors consistently retain Tm5NM1 (Stehn et al., 2006, 2013).

At least two mechanisms have been identified for the nuclear translocation of ERK. Phosphorylation by CK2 on the SPS nuclear translocation sequence of pERK is vital for both passive diffusion and active transport (Chuderland et al., 2008; Plotnikov et al., 2011). Both passive energy-independent and active nuclear transport appear to be facilitated by the association of ERK phosphorylated on the SPS site with Imp7 (Chuderland et al., 2008). In our study, WT cells were sensitive to CK2 inhibition, indicating dependence on CK2-associated phosphorylation of the SPS domain of ERK for cell proliferation. In contrast, Tm5NM1 KO MEFs were insensitive to CK2 inhibition, although phosphorylation of the SPS site of pERK is still intact. This suggests that it is the phosphorylation of the SPS nuclear translocation sequence in ERK and subsequent nuclear translocation of pSPS-pERK that is largely responsible for ERK regulation of cell proliferation in MEFs.

The results from the KO cells indicate that it is the interaction of pSPS-pERK with Imp7 that is dependent on Tm5NM1-containing actin filaments (Figure 9). There are two types of interactions that might explain this dependence on Tm5NM1. First, the interaction between pSPS-pERK and Imp7 might require Tm5NM1-containing actin filaments to facilitate their physical interaction, possibly via a scaffolding role. Second, a myosin motor might be required to physically transport pSPS-pERK to a site near the nucleus where the interaction with Imp7 can occur. We cannot discriminate between these possibilities at this time.

Knockout of Tm5NM1 was previously shown to lead to partial embryonic lethality (~50%) in two different mouse backgrounds (Hook et al., 2011), and knockout of all isoforms from the gene that produces Tm5NM1 leads to complete embryonic lethality in the preimplantation embryo (Hook et al., 2004). Furthermore, knockout of Tm5NM1 in mouse embryonic stem cells in cell culture results in complete lethality (Hook et al., 2011). It is possible that at least some of this loss of viability might be due to the effect on ERK2 signaling. Knockout of mouse ERK2 results in complete embryonic lethality (Saba-EI-Leil et al., 2003; Yao et al., 2003), and ERK2-knockout MEFs show reduced cell proliferation similar to that seen with the Tm5NM1 KO (Voisin et al., 2010).

The finding that Tm5NM1 KO MEFs proliferate at a rate substantially greater than WT cells treated with the ERK 2 and CK2 inhibitors suggests that the KO cells shifted their reliance from pERK onto alternate signal transduction pathways to drive cell growth. There are numerous reports of cross-talk within the MAPK signaling cascades and between the MAPK and PI3K pathways in the regulation of cell survival (Mendoza et al., 2011). In particular, there is strong cross-talk and compensation between the ERK/MAPK and PI3K cascades, which makes sole targeted therapy in constitutively
FIGURE 7: CK2 inhibition has no effect on the proliferation or nuclear translocation of pERK in the Tm5NM1 KO MEFs. (A) Serum-starved WT (wt/wt) and KO (ko/ko) MEFs, each from two separate mice, before (−) and after (+) 15 min serum stimulation were analyzed by Western blotting using p(SPS)-ERK, CK2, and total ERK (to evaluate protein loading) antibodies. (B) Fold induction in p(SPS)-ERK and CK2 determined after serum addition. n = 3. (C) Representative proliferation curves of serum-starved, synchronized WT (wt/wt) and Tm5NM1 KO (ko/ko) MEFs pretreated for 30 min with vehicle alone (DMSO) or CK2 inhibitor (TCMB, 0.5 μM), replated with serum, and cells counted. (D) Proliferation
activated ERK/MAPK cancers so challenging (Wee et al., 2009; Villanueva et al., 2010). Failure of signal transduction through one pathway leads to up-regulation of alternate pathways. To maintain proliferative capacity in the Tm5NM1 KO cells, multiple pathways within the complex network of signaling events must be providing a protective compensatory mechanism, albeit of reduced efficiency, due to the disrupted signal from ERK to the nucleus. That the KO cells have the same sensitivity to the MEK inhibitor UO126 as WT cells indicates that at least signaling downstream of MEK, but independent of ERK2, is likely to be involved. That Tm5NM1 KO MEFs are partially resistant to the PI3K inhibitor LY294002 might also reflect this reorganization of signal transduction pathway usage away from both the pERK and PI3K cascades to maximize the potential to proliferate.

We conclude that Tm5NM1 provides a nonredundant isoform-specific function essential for cell proliferation. This effect is mediated through the ERK/MAPK signaling cascade, where Tm5NM1 is essential for regulating CK2-dependent nuclear translocation of p-SPS-pERK to facilitate cell proliferation. Taking together the results presented in this study and the reported prevalence of the deregulation of the ERK pathway in numerous cancers (Roberts and Der, 2007), where Tm5NM1 is the predominant Tm isoform (Stehn et al., 2006), it is possible that the anti-tumor effect of the recently published anti-Tm5NM1 compound might act at least in part by inhibition of the ERK/MAPK pathway in addition to its effect on the integrity of the actin cytoskeleton (Stehn et al., 2013).

MATERIALS AND METHODS

Mice

Animal experiments were performed in accordance with UNSW Australia Animal Care and Ethics Committee approval and Australian National Health and Medical Research Council guidelines. The Tm5NM1 (F-Tg(ACoB-Tpm3.Tm5NM1)52Pgun) and Tm3 (F-Tg(ACoB-Tpm3.Tm3)70Pgun) TG mouse lines and Tm5NM1 (B6-Tpm3tm2(9d)Pgun) KO mouse line were reported previously (Bryce et al., 2003; Kee et al., 2004; Vlahovich et al., 2009). The Tm5NM1 and Tm3 TG mice (FVB/N genetic background) express the human and rat forms of Tm5NM1 and Tm3, respectively, under the control of the human β-actin promoter (Schevzov et al., 2008). The B6-Tpm3tm2(9d)Pgun mouse line is a KO for both the Tm5NM1 and Tm5NM2 isoforms. However, phenotypes in this study are attributable to lack of Tm5NM1, since Tm5NM1 is the predominant or sole isoform in the cell types examined (unpublished data). Therefore, in these studies, this mouse line is termed Tm5NM1 KO. WT control mice for TG and KO mice were age-matched, of the same background strain, and bred in the same facility, and male mice were used. The Tm4 functional null mouse line (Tpm4tm2(52P)-Pnos) was identified in an N-ethyl-N-nitrosourea mutagenesis screen. This mouse line carries a single-base pair mutation that disrupts splicing, resulting in the expression of a mutant Tm4 protein that lacks the last 24 amino acids at the C-terminus and hence cannot form a polymer along the length of the actin filament (Tobacman, 2008; Martin et al., 2010).

Cell culture

Primary MEFs were isolated from day 13.5 embryos and cultured as previously described (Schevzov et al., 2005). WT and Tm5NM1 KO MEFs were derived from mice of two genetic backgrounds: 129/SvJ (129-Tpm3tm2(9d)Pgun) and C57BL/6J Arc (B6-Tpm3tm2(9d)Pgun). WT and KO MEFs from at least two embryos of each mouse strain were analyzed. Tm5NM1-overexpressing MEFs were derived from the B6-F-Tg(ACoB-TPM3.Tm5NM1)52Pgun line, and MEFs homozygous for the transgenic locus were used for experiments. The TG/KO MEFs were derived from a cross between the Tm5NM1 TG and the Tm5NM1 KO mice on a C57BL/6J background (B6-F-Tg(ACoB-TPM3.Tm5NM1)52Pgun × B6-Tpm3tm2(9d)Pgun). Tpm4tm2(52P)-Pnos and corresponding BALB/c WT MEFs were isolated from three littermate embryos per genotype. Cultured cells were maintained in DMEM with 10% (vol/vol) fetal bovine serum (FBS) (IrvineTec, Life Technologies, Melbourne, Australia) at 37°C and 5% CO2. DNA content was analyzed by flow cytometry after propidium iodide staining (FACs Canto II flow cytometer; BD). G0/G1, S, and G2/M cell cycle phases were quantified with FACs Diva software.

Proliferation assays

MEFs were initially arrested in the G1/S phase of the cell cycle by culturing cells in DMEM containing 0.1% (vol/vol) FBS for 72 h. Cells (1 × 10^5/well) were seeded onto a 24-well plate in the presence of DMEM supplemented with 10% (vol/vol) FBS to allow reentry into the cell cycle. Cells were counted using a hemocytometer. For inhibitor studies, synchronized cells were treated with inhibitors for 30 min while suspended in trypsinization medium before plating in the presence of DMEM containing 10% (vol/vol) FBS. LY294002 (2.5 μM), U0126 (2.5 μM), ERK Activation Inhibitor Peptide 1 (2.5 μM), JNK Inhibitor I (L-Form) (1 μM), and SB202190 (10 μM) were purchased from Calbiochem, Merck Millipore, Melbourne, Australia. ERK1/2 (50 nM) and 2-(4,5,6,7-tetrahydro-2-(dimethylamino)-1H-benzol[d]imidazol-1-yl)acetic acid (TMCB) CK2 (0.5 μM) inhibitors were from Tocris Biosciences (Bristol, UK).

5-Ethynyl-2′-deoxyuridine incorporation

WT and KO MEFs were serum starved as described and seeded onto matrix-coated acrylamide hydrogels (0.03–0.3% acrylamide;certirmol, UK) onto matrix-coated acrylamide hydrogels (0.03–0.3% acrylamide;certirmol, UK). MEFs were initially arrested in the G1/S phase of the cell cycle by culturing cells in DMEM containing 0.1% (vol/vol) FBS for 72 h. Cells (1 × 10^5/well) were seeded onto a 24-well plate in the presence of DMEM supplemented with 10% (vol/vol) FBS to allow reentry into the cell cycle. Cells were counted using a hemocytometer. For inhibitor studies, synchronized cells were treated with inhibitors for 30 min while suspended in trypsinization medium before plating in the presence of DMEM containing 10% (vol/vol) FBS. LY294002 (2.5 μM), U0126 (2.5 μM), ERK Activation Inhibitor Peptide 1 (2.5 μM), JNK Inhibitor I (L-Form) (1 μM), and SB202190 (10 μM) were purchased from Calbiochem, Merck Millipore, Melbourne, Australia. ERK1/2 (50 nM) and 2-(4,5,6,7-tetrahydro-2-(dimethylamino)-1H-benzol[d]imidazol-1-yl)acetic acid (TMCB) CK2 (0.5 μM) inhibitors were from Tocris Biosciences (Bristol, UK).

Proliferation assays

MEFs were initially arrested in the G1/S phase of the cell cycle by culturing cells in DMEM containing 0.1% (vol/vol) FBS for 72 h. Cells (1 × 10^5/well) were seeded onto a 24-well plate in the presence of DMEM supplemented with 10% (vol/vol) FBS to allow reentry into the cell cycle. Cells were counted using a hemocytometer. For inhibitor studies, synchronized cells were treated with inhibitors for 30 min while suspended in trypsinization medium before plating in the presence of DMEM containing 10% (vol/vol) FBS. LY294002 (2.5 μM), U0126 (2.5 μM), ERK Activation Inhibitor Peptide 1 (2.5 μM), JNK Inhibitor I (L-Form) (1 μM), and SB202190 (10 μM) were purchased from Calbiochem, Merck Millipore, Melbourne, Australia. ERK1/2 (50 nM) and 2-(4,5,6,7-tetrahydro-2-(dimethylamino)-1H-benzol[d]imidazol-1-yl)acetic acid (TMCB) CK2 (0.5 μM) inhibitors were from Tocris Biosciences (Bristol, UK).

5-Ethynyl-2′-deoxyuridine incorporation

WT and KO MEFs were serum starved as described and seeded onto matrix-coated acrylamide hydrogels (0.03–0.3% acrylamide; certirmol, UK). MEFs were initially arrested in the G1/S phase of the cell cycle by culturing cells in DMEM containing 0.1% (vol/vol) FBS for 72 h. Cells (1 × 10^5/well) were seeded onto a 24-well plate in the presence of DMEM supplemented with 10% (vol/vol) FBS to allow reentry into the cell cycle. Cells were counted using a hemocytometer. For inhibitor studies, synchronized cells were treated with inhibitors for 30 min while suspended in trypsinization medium before plating in the presence of DMEM containing 10% (vol/vol) FBS. LY294002 (2.5 μM), U0126 (2.5 μM), ERK Activation Inhibitor Peptide 1 (2.5 μM), JNK Inhibitor I (L-Form) (1 μM), and SB202190 (10 μM) were purchased from Calbiochem, Merck Millipore, Melbourne, Australia. ERK1/2 (50 nM) and 2-(4,5,6,7-tetrahydro-2-(dimethylamino)-1H-benzol[d]imidazol-1-yl)acetic acid (TMCB) CK2 (0.5 μM) inhibitors were from Tocris Biosciences (Bristol, UK).
Mouse adipose tissue was prepared as previously described (Schevzov et al., 2008). Cell lysates were harvested in radioimmuno-precipitation assay (RIPA) buffer (20 mM Tris, pH 7.4, 150 mM sodium chloride, 1% [vol/vol] Nonidet P-40, 0.5% [vol/vol] sodium deoxycholate, 1 mM EDTA, 0.1% [vol/vol] SDS, Complete Mini Protease Inhibitor EDTA free tablet [Roche Diagnostics], and PhosSTOP phosphatase inhibitor cocktail tablet [Roche Diagnostics]). Antibodies used included α/9d (mouse monoclonal; Tm1, 2, 3, 5a, 5b, 6), γ/9d (sheep polyclonal; Tm5NM1; Schevzov et al., 2005), LC1 (mouse and human Tm5NM1; Sung et al., 2000), CG3 (Novy et al., 1993), δ/9d (rabbit polyclonal; Tm4; Hannan et al., 1998), and δ/1b (mouse monoclonal; Tm4; Schevzov et al., 2011). Also used were phospho–p44/p42 MAPK (ERK1/2; Thr-202/Tyr-204), total p44/p42 MAPK (ERK1/2) and cyclin D1 (A-12) antibodies (Cell Signaling Technology, via Genesearch, Brisbane, Australia), anti–phospho-SPS-ERK (obtained from Rony Seger, Weizmann Institute of Science, Rehovot, Israel), α-tubulin (clone DM 1A) antibody (Sigma-Aldrich, Sydney, Australia) and Imp7 IPO7 monoclonal antibody (M07), clone 4G6 (Sapphire Biosciences, Sydney, Australia). Anti-rabbit, anti-sheep, and anti-mouse immunoglobulin G–conjugated horseradish peroxidase secondary antibodies were from GE Healthcare, Sydney, Australia. Western blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Body fat and adipose tissue analysis

After starvation for 14-16 h, total body fat and lean tissue composition of male mice (14–15 wk old) was assessed using a Mouse GE Lunar PIXImus densitometer (GE Healthcare; MacArthur et al., 2008). DNA content of epididymal adipose tissue of 14- to 15-wk-old mice after 14–16 h of starvation (n = 8/group) was determined as previously reported (Labarca and Paigen, 1980).

![Image of Western blotting results](image-url)

**Western blotting**

Mouse adipose tissue was prepared as previously described (Schevzov et al., 2008). Cell lysates were harvested in radioimmuno-precipitation assay (RIPA) buffer (20 mM Tris, pH 7.4, 150 mM sodium chloride, 1% [vol/vol] Nonidet P-40, 0.5% [vol/vol] sodium deoxycholate, 1 mM EDTA, 0.1% [vol/vol] SDS, Complete Mini Protease Inhibitor EDTA free tablet [Roche Diagnostics], and PhosSTOP phosphatase inhibitor cocktail tablet [Roche Diagnostics]). Antibodies used included α/9d (mouse monoclonal; Tm1, 2, 3, 5a, 5b, 6), γ/9d (sheep polyclonal; Tm5NM1; Schevzov et al., 2005), LC1 (mouse and human Tm5NM1; Sung et al., 2000), CG3 (Novy et al., 1993), δ/9d (rabbit polyclonal; Tm4; Hannan et al., 1998), and δ/1b (mouse monoclonal; Tm4; Schevzov et al., 2011). Also used were phospho–p44/p42 MAPK (ERK1/2; Thr-202/Tyr-204), total p44/p42 MAPK (ERK1/2) and cyclin D1 (A-12) antibodies (Cell Signaling Technology, via Genesearch, Brisbane, Australia), anti–phospho-SPS-ERK (obtained from Rony Seger, Weizmann Institute of Science, Rehovot, Israel), α-tubulin (clone DM 1A) antibody (Sigma-Aldrich, Sydney, Australia) and Imp7 IPO7 monoclonal antibody (M07), clone 4G6 (Sapphire Biosciences, Sydney, Australia). Anti-rabbit, anti-sheep, and anti-mouse immunoglobulin G–conjugated horseradish peroxidase secondary antibodies were from GE Healthcare, Sydney, Australia. Western blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Body fat and adipose tissue analysis

After starvation for 14-16 h, total body fat and lean tissue composition of male mice (14–15 wk old) was assessed using a Mouse GE Lunar PIXImus densitometer (GE Healthcare; MacArthur et al., 2008). DNA content of epididymal adipose tissue of 14- to 15-wk-old mice after 14–16 h of starvation (n = 8/group) was determined as previously reported (Labarca and Paigen, 1980).

![Image of Western blotting results](image-url)

**Western blotting**

Mouse adipose tissue was prepared as previously described (Schevzov et al., 2008). Cell lysates were harvested in radioimmuno-precipitation assay (RIPA) buffer (20 mM Tris, pH 7.4, 150 mM sodium chloride, 1% [vol/vol] Nonidet P-40, 0.5% [vol/vol] sodium deoxycholate, 1 mM EDTA, 0.1% [vol/vol] SDS, Complete Mini Protease Inhibitor EDTA free tablet [Roche Diagnostics], and PhosSTOP phosphatase inhibitor cocktail tablet [Roche Diagnostics]). Antibodies used included α/9d (mouse monoclonal; Tm1, 2, 3, 5a, 5b, 6), γ/9d (sheep polyclonal; Tm5NM1; Schevzov et al., 2005), LC1 (mouse and human Tm5NM1; Sung et al., 2000), CG3 (Novy et al., 1993), δ/9d (rabbit polyclonal; Tm4; Hannan et al., 1998), and δ/1b (mouse monoclonal; Tm4; Schevzov et al., 2011). Also used were phospho–p44/p42 MAPK (ERK1/2; Thr-202/Tyr-204), total p44/p42 MAPK (ERK1/2) and cyclin D1 (A-12) antibodies (Cell Signaling Technology, via Genesearch, Brisbane, Australia), anti–phospho-SPS-ERK (obtained from Rony Seger, Weizmann Institute of Science, Rehovot, Israel), α-tubulin (clone DM 1A) antibody (Sigma-Aldrich, Sydney, Australia) and Imp7 IPO7 monoclonal antibody (M07), clone 4G6 (Sapphire Biosciences, Sydney, Australia). Anti-rabbit, anti-sheep, and anti-mouse immunoglobulin G–conjugated horseradish peroxidase secondary antibodies were from GE Healthcare, Sydney, Australia. Western blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Body fat and adipose tissue analysis

After starvation for 14-16 h, total body fat and lean tissue composition of male mice (14–15 wk old) was assessed using a Mouse GE Lunar PIXImus densitometer (GE Healthcare; MacArthur et al., 2008). DNA content of epididymal adipose tissue of 14- to 15-wk-old mice after 14–16 h of starvation (n = 8/group) was determined as previously reported (Labarca and Paigen, 1980).

![Image of Western blotting results](image-url)

**Western blotting**

Mouse adipose tissue was prepared as previously described (Schevzov et al., 2008). Cell lysates were harvested in radioimmuno-precipitation assay (RIPA) buffer (20 mM Tris, pH 7.4, 150 mM sodium chloride, 1% [vol/vol] Nonidet P-40, 0.5% [vol/vol] sodium deoxycholate, 1 mM EDTA, 0.1% [vol/vol] SDS, Complete Mini Protease Inhibitor EDTA free tablet [Roche Diagnostics], and PhosSTOP phosphatase inhibitor cocktail tablet [Roche Diagnostics]). Antibodies used included α/9d (mouse monoclonal; Tm1, 2, 3, 5a, 5b, 6), γ/9d (sheep polyclonal; Tm5NM1; Schevzov et al., 2005), LC1 (mouse and human Tm5NM1; Sung et al., 2000), CG3 (Novy et al., 1993), δ/9d (rabbit polyclonal; Tm4; Hannan et al., 1998), and δ/1b (mouse monoclonal; Tm4; Schevzov et al., 2011). Also used were phospho–p44/p42 MAPK (ERK1/2; Thr-202/Tyr-204), total p44/p42 MAPK (ERK1/2) and cyclin D1 (A-12) antibodies (Cell Signaling Technology, via Genesearch, Brisbane, Australia), anti–phospho-SPS-ERK (obtained from Rony Seger, Weizmann Institute of Science, Rehovot, Israel), α-tubulin (clone DM 1A) antibody (Sigma-Aldrich, Sydney, Australia) and Imp7 IPO7 monoclonal antibody (M07), clone 4G6 (Sapphire Biosciences, Sydney, Australia). Anti-rabbit, anti-sheep, and anti-mouse immunoglobulin G–conjugated horseradish peroxidase secondary antibodies were from GE Healthcare, Sydney, Australia. Western blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Body fat and adipose tissue analysis

After starvation for 14-16 h, total body fat and lean tissue composition of male mice (14–15 wk old) was assessed using a Mouse GE Lunar PIXImus densitometer (GE Healthcare; MacArthur et al., 2008). DNA content of epididymal adipose tissue of 14- to 15-wk-old mice after 14–16 h of starvation (n = 8/group) was determined as previously reported (Labarca and Paigen, 1980).
**Illumina BeadArray analysis**

Gene expression profiling was performed on epididymal adipose tissue from Tm5NM1 TG and WT control mice (14- to 16-h–fasted, 4-mo-old male mice; n = 5 and 4/group, respectively) using Illumina (Scoresby) 46K mouse BeadArrays as previously outlined (Pearen et al., 2009). RNA was purified using a mini-RNeasy kit (Qiagen, Sydney, Australia) according to the manufacturer’s instructions. Integrity of the total RNA samples was assessed using the Agilent Bioanalyzer 2100, and RNA integrity scores >7.8 were present in all samples. Amplified cRNA (1.5 μg) was hybridized to Sentic Mouse-6.v1 BeadChip arrays (Illumina, San Diego, CA). BeadChip arrays (Accession GPL6283) were scanned with an Illumina BeadStation Scanner and the data imported into Gene-Spring GX v7.3.1 software (Agilent, Santa Clara, CA) for data analysis. Data were normalized to control genes, genes with an Illumina detection score equal to 1, and all probes except the genes with an Illumina detection score equal to 1 were filtered out to remove probes without adequate expression levels. A parametric Welch test (unequal variance) was performed (p cut-off of 0.05), and multiple testing correction (Benjamini and Hochberg false discovery rate) was then applied to genes that passed the Welch test (PASW Statistics 18 software; SPSS). After the statistical filtering, 70 genes were found to be changed by ≥2.0-fold and 483 by ≥1.75-fold. Ingenuity Pathway Analysis (www.ingenuity.com) was performed on genes with positive or negative fold changes between TG and WT tissue of >1.5 (1283 transcripts).

**qRT-PCR analysis**

RNA was extracted from epididymal adipose tissue using TRI-Reagent (Sigma-Aldrich) and further purified using a mini-RNeasy kit (Qiagen). cDNA was synthesized from 1 μg of total RNA using M-MLV Reverse Transcriptase (Promega, Sydney, Australia) primed by random hexamers, according to the manufacturer’s instructions. qRT-PCR was performed on epididymal adipose tissue (n = 10/group). The primers were either purchased from Qiagen or designed using the Primer 3 Input (version 4.0) or Primer-Blast (National Center for Biotechnology Information); see Supplemental Table S1 for primer sequences. PCR was performed in a final volume of 20 μl, consisting of diluted cDNA sample, 1× Brilliant II SYBR Green QPCR Master Mix (Stratagene, via Integrated Sciences, Sydney, Australia), primers optimized for each target gene, and nuclease-free water. qRT-PCR was performed using a Mx300P machine (Stratagene) and was conducted over 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min preceded by an initial 95°C for 10 min. Relative quantities of target transcripts were calculated from triplicate samples after normalization of the data against HPRT using the standard curve method. For each target gene, qRT-PCR was performed at least twice. TG versus WT mice were compared using two-tailed Student’s t test assuming unequal variances.

**Immunofluorescence**

MEFs were plated onto glass chamber slides and immunostained with pERK1/2 (Aplin et al., 2001), followed by donkey/anti-rabbit/555 antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Phospho–Elk-1 (Ser-383) (Cell Signaling Technology) immunostaining was conducted after fixation of the cells in 4% (vol/vol) paraformaldehyde and blocking of nonspecific binding; dilution of the antibody (1/500) was in 1% (vol/vol) bovine serum albumin (BSA), 10% (vol/vol) normal goat serum, and 0.1% (vol/vol) Triton X-100. The cytochalasin D (Sigma-Aldrich) experiment was conducted by first seeding and culturing cells for 24 h, serum starving them for 24 h, and treating them for 90 min with cytochalasin D (500 nM) or dimethyl sulfoxide (DMSO). MEFs were fixed and stained with phalloidin ATTO 488 (ATTO-TEC, Siegen, Germany) as previously described (Schevzov et al., 2008). Coverslips were mounted using FluorSave Reagent (Calbiochem). Images were captured with a Zeiss Axioskop 40 FL (Carl Zeiss, Oberkochen, Germany) using the 40×/0.65 numerical aperture (NA) or 10×/0.25NA objectives and photographed using a Zeiss AxioCam MRC charge-coupled device (CCD) camera.

Kidneys were dissected from 4-wk-old Tm5NM1 TG (n = 5), WT (n = 4), and KO (n = 8) mice, all on a C57BL6 background. Kidneys were fixed in 10% neutral buffered Formalin and embedded in paraffin using an Excelsior tissue processor (Thermo Scientific, Melbourne, Australia) and sectioned at 4 μm. Antigen retrieval was performed in 10 mM citrate buffer, pH 5.8, in an RHS-1 microwave
vacuum histoprocessor (Milestone, Kalamazoo, MI) at 120°C. Sections were blocked in 5% goat serum, 5% FBS, and 1% BSA for 2 h at room temperature and incubated overnight at room temperature with a Ki67 rabbit polyclonal antibody (SP6, 1:200; Novus Biologicals). Secondary antibody (1:750; Immunoresearch Laboratories) coupled with the fluorochrome Alexa Fluor 555 was diluted in blocking buffer. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI; 1:10,000, Molecular Probes, Life Technologies, Melbourne, Australia). Images were captured with a BX51 fluorescence microscope equipped with a DP73 CCD color camera (Olympus). Ki67-positive nuclei in kidney cortical tubules were counted and normalized to total nuclei (DAPI stained). Nuclei were counted in three random fields (2000 nuclei/field) per kidney where proliferation was evident using ImageJ software.

**Proximity ligation assay**

Intracellular protein–protein interactions were examined using the Duolink PLA kit (Olink Biosciences, Sigma-Aldrich). In brief, cells were cultured and serum starved as described, fixed with 4% paraformaldehyde, and permeabilized with 0.1% (vol/vol) Triton X-100 in phosphate-buffered saline. Primary antibodies were incubated overnight at 4°C and samples processed according to the instructions provided. The red PLA signal data were visualized using a Zeiss Axioskop 40 FL (Carl Zeiss), photographed using a Zeiss AxioCam MRc CCD camera, and quantitated using ImageJ.

**Statistical analysis**

Statistical analysis of the BeadArray data was as described. All other experimental data are expressed as mean ± SEM for n > 3 experiments. Provided the data passed the D'Agostino and Perron test for Gaussian distribution (PASW Statistics 18), significant difference between groups was determined by a two-tailed Student's t test assuming unequal variances for comparisons between two groups or by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc for more than two groups. If data did not fit a Gaussian distribution or group sizes were small (n < 5), the nonparametric Kruskal–Wallis test was performed to determine significance between groups. For intergroup comparisons of greater than two, a Dunn multiple comparison test was performed (PASW Statistics). p < 0.05 was considered statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

**ACKNOWLEDGMENTS**

We thank Renee Szokolai, Melissa Desouza, and Shane Whitaker for technical assistance and Nicole Bryce for critical reading of the manuscript. This work was supported by Project Grants APP1004175 and APPS70762 from the Australian National and Medical Research Council (P.W.G., G.S., and E.C.H. and A.J.K., respectively) and funding from the Kids Cancer Project. A.J.K. and J.R.S. are Kids Cancer Project, Childhood Cancer Cytoskeletal Consortium (C4) Research Fellows. J.D.C. was supported by the Medical Foundation of the University of Sydney, E.A.M. was a Cancer Institute NSW Fellow. P.W.G. was a Principal Research Fellow of the Australian National and Medical Research Council (163626). B.T.K. was supported by a Program Grant (1016647), an Independent Research Institutes Support Scheme Grant (361646) from the National and Medical Research Council, and a fellowship from the Sylvia and Charles Viertel Foundation. I.P. was supported by a fellowship from the Deutsche Forschungsgemeinschaft. This work was also supported by the Australian Cancer Research Fund and a Victorian State Government Operational Infrastructure Support Grant.
Huang S, Ingber DE (2002). A discrete cell cycle checkpoint in late G1(1) that is cytoskeleton-dependent and MAP kinase (Erk)-independent. Exp Cell Res 275, 255–264.

Johnson M, East DA, Mulvihill DP (2014). Formins determine the functional properties of actin filaments in yeast. Curr Biol 24, 1525–1530.

Jongstra-Bilen J, Jongstra J (2006). Leucocyte-specific protein 1 (LSP1): a regulator of leucocyte emigration in inflammation. Immunol Res 35, 65–74.

Kee AJ, Schevazov G, Nair-Shalliker V, Robinson CS, Vrhovski B, Ghoddui M, Qiu MR, Lin JJ, Weinberger R, Gunning PW, et al. (2004). Sorting of a nonmuscle tropomyosin to a novel cytoskeletal compartment in skeletal muscle results in muscular dystrophy. J Cell Biol 166, 691–706.

Klein EA, Yin L, Kothapalli D, Castagnino P, Byfield FJ, Xu T, Levental I, Hawthorne E, Janney PA, Assoian RK (2009). Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening. Curr Biol 19, 1511–1518.

Klein EA, Yung Y, Castagnino P, Kothapalli D, Assoian RK, David AC (2007). Cell adhesion, cellular tension, and cell cycle control. Methods Enzymol 426, 155–175.

Labarca C, Paigen K (1980). A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 102, 344–352.

Leinweber BD, Leavis PC, Grabarek Z, Wang CL, Morgan KG (1999). Extra-cellular regulated kinase (ERK) interaction with actin and the calponin homology (CH) domain of actin-binding proteins. Biochem J 344, 117–123.

Lemmon MA, Sardet C, Pages G, L’Allemain G, Brunet A, Pouyssegur J (1993). Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapk) in fibroblasts. J Cell Biol 122, 1079–1088.

Lin JJ, Helfman DM, Hughes SH, Chou CS (1985). Tropomyosin isoforms in chicken embryo fibroblasts: purification, characterization, and changes in Rous sarcoma virus-transformed cells. J Cell Biol 100, 697–703.

MacArthur DG, Seto JT, Chan S, Quinlan KG, Raftery JM, Turner N, Nicholson MD, Kee AJ, Hardeman EC, Gunning PW, et al. (2008). An Actn3 knockout mouse provides mechanistic insights into the association between alpha-actinin-3 deficiency and human athletic performance. Hum Mol Genet 17, 1076–1086.

Martin C, Schevazov G, Gunning P (2010). Alternatively spliced N-terminal exons in tropomyosin isoforms do not act as autonomous targeting signals. J Struct Biol 170, 286–293.

Mc Nicol A, Shibou TS, Pampolina C, Israelis SJ (2001). Incorporation of map kinases into the platelet cytoskeleton. Thromb Res 103, 25–34.

Mendoza MC, Et ER, Blenis J (2011). The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. Trends Biochem Sci 36, 320–328.

Miecznikowski JB, Drubin DG (2011). Building distinct actin filament networks in a single cell by coupling in vivo contractility and inter-phase ligation. Nat Methods 3, 995–1000.

Nithianandaram-Jones GN, Wilm B, Goldring CE, Muller J, Cross MJ (2012). ERKs: structure, regulation and function. Cell Signal 24, 2187–2196.

Nowy RE, Sellers JR, Liu LF, Lin JJ (1993). In vitro functional characterization of bacterially expressed human fibroblast tropomyosin isoforms and their chimeric mutants. Cell Motil Cytoskel 26, 248–261.

O’Neill GM, Stenh J, Gunning PW (2008). Tropomyosins as interpreters of the signalling environment to regulate the local cytoskeleton. Semin Cancer Biol 18, 35–44.

Pearen MA, Ryall JG, Lynch GS, Muscat GE (2009). Expression profiling of skeletal muscle following acute and chronic beta2-adrenergic stimulation: implications for hypertrophy, metabolism and circadian rhythm. BMC Genomics 10, 448.

Pericval JM, Thomas G, Cocket TA, Gardiner EM, Jeffrey PL, Lin JJ, Weinberger RP, Gunning P (2000). Sorting of tropomyosin isoforms in a common cytoplasm. Curr Biol 21, R560–R569.

Potter RJ, Kool J, Wester K, Hydbring P, Bahram F, Larsson LG, et al. (2006). Direct demonstration of individual endogenous protein complexes in situ by proximity ligation. Nat Methods 3, 995–1000.

Rudd PF, Haas NK, Bonello T, Desouza M, Kottyan G, Treutlein H, Zeng SL, Meloche S (2003). An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development. EMBO Rep 4, 964–968.

Schevazov G, Fath T, Vrhovski B, Vlahovic N, Rajan S, Hook J, Joye JA, Lembert F, Puttur F, Lin JJ, et al. (2008). Divergent regulation of the sarcomere and the cytoskeleton. J Biol Chem 283, 275–283.

Schevazov G, Lloyd C, Gunning P (1992). High level expression of transfected beta-and-gamma-actin genes differentially impacts on myoblast cytotarchitecture. J Cell Biol 117, 775–785.

Schevazov G, Lloyd C, Hallststones D, Gunning P (1993). Differential regulation of tropomyosin isoform organisation and gene expression in response to altered actin gene expression. J Cell Biol 121, 811–821.

Schevazov G, Vrhovski B, Bryce NS, Elmir S, Qiu MR, O’Neill GM, Yang N, Verrills NM, Kavallahs M, Gunning PW (2005). Tissue-specific tropomyosin isoform composition. J Histochem Cytochem 53, 557–570.

Schrick C, Fischer A, Srivastava DP, Tronson NC, Penzes P, Radulovic J (2007). N-cadherin regulates cytoskeletonally associated IQGAP1/ERK signaling and memory formation. Neuron 55, 786–798.

Schwartz MA, Assoian RK (2001). Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. J Cell Sci 114, 2553–2560.

Seger R, Koles SA (1998). The MAPK signaling cascade. FASEB J 9, 726–735.

Skau CT, Neidt EM, Kovar DR (2009). Role of tropomyosin in formin-mediated contractile ring assembly in fission yeast. Mol Biol Cell 20, 2160–2173.

Smith ER, Snedberg JL, Rula ME, Xu XX (2004). Regulation of Ras-MAPK pathway mitogenic activity by restricting nuclear entry of activated MAPK in endothelial differentiation of embryonic carcinoma and stem cells. J Cell Biol 164, 689–699.

Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, Wester K, Hydbring P, Bahram F, Larsson LG, et al. (2006). Direct demonstration of individual endogenous protein complexes in situ by proximity ligation. Nat Methods 3, 995–1000.

Stehn JR, Haas NK, Bonello T, Desouza M, Kottyan G, Treutlein H, Zeng SL, Meloche S (2003). An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development. EMBO Rep 4, 964–968.

Tobacman LS (2008). Cooperative binding of tropomyosin to actin. Cell Mol Life Sci 64, 85–94.

Vetterkind S, Poythress RH, Lin QQ, Morgan KG (2013). Hierarchical scaffolding of an ERK1/2 activation pathway. Cell Commun Signal 11, 65.

Villanueva J, Valtur L, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Ip C, Wu J, Gunning PW, et al. (2004). Acquired resistance to BFA4 inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/Pi3K. Cancer Cell 18, 683–695.

Vlahovic N, Kee AJ, Van der Poel C, Kettele E, Hernandez-Devezie D, Lucas C, Lynch GS, Parton RG, Gunning PW, Hardeman EC (2009). Cytoskeletal tropomyosin TmSNM1 is required for normal excitation-contraction coupling in skeletal muscle. Mol Biol Cell 20, 400–409.
Welsh CF, Roovers K, Villanueva J, Liu Y, Schwartz MA, Assoian RK (2001). Timing of cyclin D1 expression within G1 phase is controlled by Rho. Nat Cell Biol 3, 950–957.

White CD, Brown MD, Sacks DB (2009). IQGAPs in cancer: a family of scaffold proteins underlying tumorigenesis. FEBS Lett 583, 1817–1824.

Yao Y, Li W, Wu J, Germann UA, Su MS, Kuida K, Boucher DM (2003). Extracellular signal-regulated kinase 2 is necessary for mesoderm differentiation. Proc Natl Acad Sci USA 100, 12759–12764.

Yoon S, Seger R (2006). The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors 24, 21–44.

Voisin L, Saba-El-Leil MK, Julien C, Fremin C, Meloche S (2010). Genetic demonstration of a redundant role of extracellular signal-regulated kinase 1 (ERK1) and ERK2 mitogen-activated protein kinases in promoting fibroblast proliferation. Mol Cell Biol 30, 2918–2932.

Weber JD, Raben DM, Phillips PJ, Baldassare JJ (1997). Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. Biochem J 326, 61–68.

Wee S, Jagani Z, Xiang KX, Loo A, Dorsch M, Yao YM, Sellers WR, Lengauer C, Stegmeier F (2009). PI3K pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers. Cancer Res 69, 4286–4293.