Aging Cell (2012) **11,** pp262–268 Doi: 10.1111/j.1474-9726.2011.00782.x

CCAAT-enhancer-binding protein-beta expression *in vivo* is associated with muscle strength

Lorna W. Harries,[1] Luke C. Pilling,[2] L. Dena G. Hernandez,[3] Rachel Bradley-Smith,[1] William Henley,[2,4] Andrew B. Singleton,[3] Jack M. Guralnik,[5] Stefania Bandinelli,[6] Luigi Ferrucci[7] and David Melzer[1]

[1]Institute of Biomedical and Clinical Sciences, Peninsula College of Medicine and Dentistry, University of Exeter, Exeter EX1 2LU, UK
[2]Epidemiology and Public Health, Peninsula College of Medicine and Dentistry, University of Exeter, Exeter EX1 2LU, UK
[3]Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, MD 20892, USA
[4]School of Mathematics and Statistics, University of Plymouth, Plymouth, UK
[5]Laboratory of Epidemiology, Demography and Biometry, National Institute on Aging, Bethesda, MD, USA
[6]Geriatric Unit, Azienda Sanitaria di Firenze, Florence, Italy
[7]National Institute on Aging, Baltimore, MD, USA

Summary

Declining muscle strength is a core feature of aging. Several mechanisms have been postulated, including CCAAT/enhancer-binding protein-beta (C/EBP-β)-triggered macrophage-mediated muscle fiber regeneration after micro-injury, evidenced in a mouse model. We aimed to identify *in vivo* circulating leukocyte gene expression changes associated with muscle strength in the human adult population. We undertook a genome-wide expression microarray screen, using peripheral blood RNA samples from InCHIANTI study participants (aged 30 and 104). Logged expression intensities were regressed with muscle strength using models adjusted for multiple confounders. Key results were validated by real-time PCR. The Short Physical Performance Battery (SPPB) score tested walk speed, chair stand, and balance. *CEBPB* expression levels were associated with muscle strength (β coefficient = 0.20560, $P = 1.03*10^{-6}$, false discovery rate $q = 0.014$). The estimated handgrip strength in 70-year-old men in the lowest *CEBPB* expression tertile was 35.2 kg compared with 41.2 kg in the top tertile. *CEBPB* expression was also associated with hip, knee, ankle, and shoulder strength and the SPPB score ($P = 0.018$). Near-study-wide associations were also noted for *TGF-β3* ($P = 3.4*10^{-5}$, $q = 0.12$) and *CEBPD* expression ($P = 9.7*10^{-5}$, $q = 0.18$) but not for *CEBPA* expression. We report here a novel finding that raised *CEBPB* expression in circulating leukocyte-derived RNA samples *in vivo* is associated with greater muscle strength and better physical performance in humans. This association may be consistent with mouse model evidence of *CEBPB*-triggered muscle repair: if this mechanism is confirmed, it may provide a target for intervention to protect and enhance aging muscle.

Key words: inflammation; macrophage; mechanism; population; regeneration; transcription.

Correspondence
Professor David Melzer, Peninsula Medical School, Barrack Road, Exeter EX2 5DW, UK. Tel.: +44-0139-240-6751; fax: 44-1392-406767; e-mail: david.melzer@pms.ac.uk

Accepted for publication *4 December 2011*

Introduction

Aging is associated with a gradual loss of muscle mass and strength (Lang *et al.*, 2009) accompanied by increasing susceptibility to injury and decreased capacity to repair and regenerate muscle (Peake *et al.*, 2010). Severe muscle loss (or 'sarcopenia') can eventually result in impairments and limitations to physical functioning. Faulkner *et al.* (1995) argued that 'everyday' contraction-induced injury contributed to muscle aging (Faulkner *et al.*, 1990). Other suggested causes for sarcopenia include loss of motor unit and local motor neuron changes, but also pathways with systemic elements such as hormone/growth factor signaling, and inflammation (Lang *et al.*, 2009).

Muscle repair after injury or change of use starts with an initial acute inflammatory response (typically from 0 to 48 h) followed by a repair and regeneration phase, typically lasting from 48 h to 10 days (Tidball, 2005). Following injury, local muscle tissue secretes pro-inflammatory (Th1) cytokines (in particular TNF-α and IFN-γ, but also IL-1β, IL-6 and others; see Fig. 1a for simplified representation of the pathways from the published literature) that instigate an innate immune response. Neutrophil invasion enhances inflammation at the injury site (Nguyen & Tidball, 2003; Tidball, 2005) and is followed by local migration of macrophages, monocytes (St Pierre & Tidball, 1994), and T-helper type 1 (Th1) lymphocytes. Monocytes are then available to differentiate into specific macrophage subtypes (Arnold *et al.*, 2007), which are essential for effective muscle regeneration (Robertson *et al.*, 1993).

At the late stage of the pro-inflammatory phase, there is increased production of IL-4, IL-13, and transforming growth factor-beta (TGF-β) from T-helper type 2 (Th2) lymphocytes; phagocytosis of necrotic tissue also influences this change in phenotype (Arnold *et al.*, 2007). These cytokines inhibit the production of Th1 cytokines and initiate the accumulation of alternatively activated (anti-inflammatory/M2) macrophages, which include wound-healing and regulatory phenotypes (Mosser & Edwards, 2008), thereby promoting tissue repair (Villalta *et al.*, 2009; Fig. 1b). As inflammation is attenuated, myoblast differentiation is induced and new muscle fibers are formed.

CCAAT-enhancer-binding protein-beta (C/EBP-β) has a pivotal role in these processes. Ruffell *et al.* (2009) showed in a mouse model that deletion of two CREB-binding sites from the *CEBPB* promoter prevented C/EBP-β induction and prevented the transition from M1 to M2 macrophage-specific gene expression during muscle repair. In their model, the pro-inflammatory response was unaffected, but muscle fiber regeneration was severely compromised, with the injured area showing a fibrotic appearance, with fewer, smaller, and calcified myofibers. Although *CEBPB* is expressed at basal levels in M1 macrophages, significant (typically 10-fold) up-regulation of *CEBPB* above constitutive levels is required for M2 activation, and therefore muscle repair (Bradley *et al.*, 2003).

Another pathway suggested as pivotal in muscle regeneration is Wnt signaling; age-related loss of muscle strength caused by increased fibrosis was associated with increased systemic Wnt signaling in aged mice (Brack *et al.*, 2007). Conversely, others have found that Wnt signaling induced the myogenic specification of resident myeloid stem cells during muscle regeneration (Polesskaya *et al.*, 2003); therefore, it is likely that an optimal trade-off may be reached, but that it changes over the lifespan of an organism.

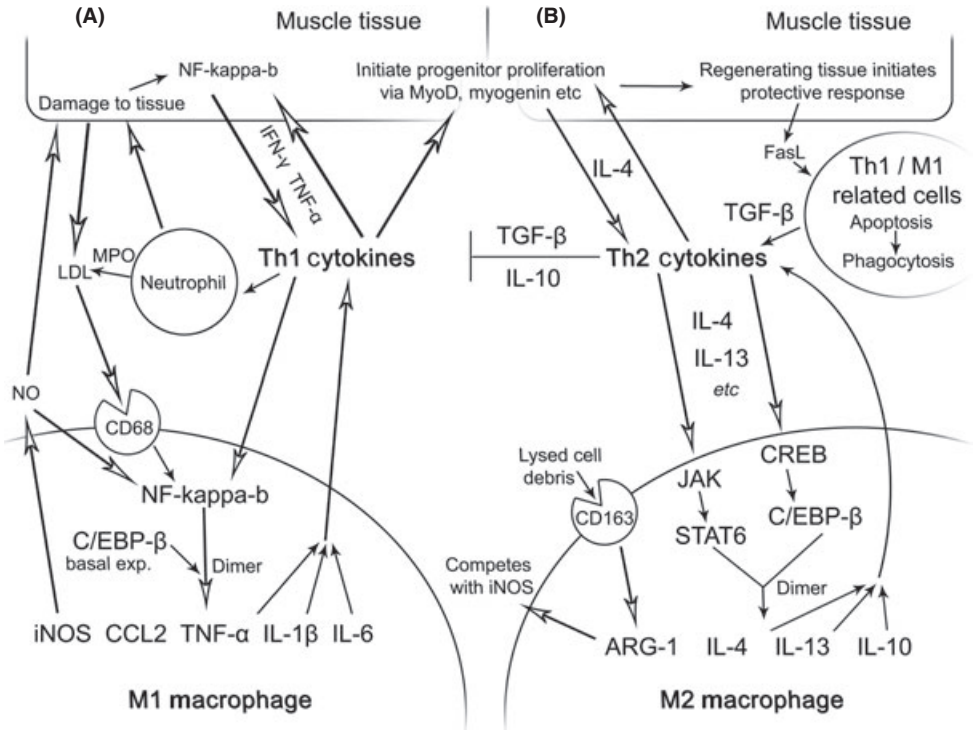

Fig. 1 Macrophage polarization during muscle injury/repair. Macrophages have a repertoire of phenotypes ranging from classical activation to several alternative activation states. These are generally termed pro-inflammatory/M1 (a) and anti-inflammatory/M2 (b), respectively. They are functionally distinct, and the correct differentiation at the right time is critical to an effective regeneration process. Drawn from the literature, this diagram represents that main factors involved in the classical (a) and alternative activation states (b) of macrophages as the time from wounding progresses. The review by Tidball & Villalta (2010) was particularly informative. LDL, low-density lipid; Th1, T-helper type 1/pro-inflammatory response; Th2, T-helper type 2/anti-inflammatory response.

While muscle repair has been studied in depth '*in vitro*' and in animal models, '*in vivo*' human studies are intrinsically more difficult. In theory, gene expression patterns could be studied in serial muscle biopsies, but this would be too invasive and would interfere with repair processes. In this study, we instead examined expression in blood (leukocyte) samples collected with immediate stabilization of RNA, so that results reflect *in vivo* mRNA expression. Blood-derived white cell transcriptome studies have already proved valuable in identifying signatures of major diseases and drug responses (Dumeaux *et al.*, 2010). Studying systemic factors in muscle strength may be particularly informative, as experimental transplantation of old muscle to a young rat host results in regeneration as marked as that seen in young muscle, suggesting that circulating factors may be critical (Carlson & Faulkner, 1989).

In this study, we aimed to test the relevance of *CEBPB* and other transcripts to muscle strength *in vivo*. The InCHIANTI population study (http://www.inchiantistudy.net) is particularly useful for this as it includes a wide age range and muscle strength measures at multiple sites.

Results

Six hundred and ninety-eight people were included in the analysis, ranging in age from 30 to 104 years (Table 1). Nearly 45% of the sample was men, and 54% had been non-smokers. In linear models adjusted for multiple potential confounders (see Experimental procedures), *CEBPB* transcript expression emerged to be most strongly associated with handgrip muscle strength: β coefficient = 0.20560, $P = 1.03*10^{-6}$ (Table 2; for table of top 250 regression results, see Table S1). *CEBPB* was also the only transcript to reach study-wide significance, accounting for multiple statistical testing of array data [false discovery rate (FDR) *q*-value = 0.014]. Associations with muscle strength for six other transcripts were likely to be robust (i.e., with a FDR *q*-value < 0.2, indicating that more than 80% of these are valid associations). Notable in these was *TGF-β3* ($P = 3.4*10\text{-}5$, $q = 0.12$) and *CEBPD* expression ($P = 9.7*10^{-5}$, $q = 0.18$). Relatively little is known about the four other transcripts *(TAAR8*, *EXOC1*, *CBWD5*, and *CA433556–CD40L)*. There were no associations with other measured *CEBP*-transcripts, namely *CEBPA*, *CEBPZ*, *CEBPG,* and *CEBPE* (for all $P > 0.18$).

CEBPB expression and grip strength: effect sizes

We focused on *CEBPB* expression, being the only gene to reach study-wide significance with muscle strength. First, we explored the associations between *CEBPB* expression and white blood cell counts. In regression models adjusted for the same terms as previously but testing each white cell subtype separately, we found a strong negative association between *CEBPB* expression and increasing lymphocyte percentage (standardized β = −0.50, $P < 0.001$) and a marginally less strong positive association with increasing neutrophil percentage (standardized β = 0.47, $P < 0.001$). There was no association with circulating monocyte percentage. We tested the model for interactions between *CEBPB* expression and lymphocyte percentage for handgrip muscle strength: the interaction term was not significant ($P = 0.250$).

To provide estimates of the unadjusted effect sizes, we show the distribution of handgrip muscle strength by tertile of *CEBPB* expression in men and women separately (Fig. 2) for those in the middle tertile of both neutrophil and lymphocyte percentages ($n = 160$). Using our fully adjusted models, we estimated grip strength for 70-year-old men or women (Table 3). This showed similar results to the unadjusted data, with mean handgrip strength in men in the highest *CEBPB* expression tertile being 41.2 kg compared with 35.2 kg in the bottom tertile. In women, there was a similar spread of predicted mean grip strength (from 26.0 to 20.0 kg, respectively).

Advancing age is associated with lower handgrip strength (β = −0.5, $P < 1E\text{-}10$) and separately with lower *CEBPB* expression (β = −0.15, $P < 1E\text{-}4$); however, age (β = −0.04, $P > 0.05$) ceases to be significantly

Table 1 Characteristics of the study sample

Study sample characteristics	*n* (%)
Age (years)	
30–65	163 (23.35)
66–85	444 (63.61)
86–104	91 (13.04)
Gender	
Men	313 (44.84)
Women	385 (55.16)
Site	
Greve	342 (49)
Bagno a Ripoli	356 (51)
Education	
None/elementary	416 (59.61)
Secondary	93 (13.32)
High school	87 (12.46)
University/professional	102 (14.61)
Pack-years smoked (lifetime)	
None	379 (54.30)
< 20	153 (21.92)
20–39	101 (14.47)
40+	55 (7.88)
Missing	10 (1.43)
Waist circumference (cm), Mean (SD)	698, 95.3 (12.1)
Leukocyte fraction (%)	**Mean ± SD**
Neutrophils	57.5 ± 9.14
Lymphocytes	30.8 ± 8.66
Monocytes	7.97 ± 2.13
Eosinophils	3.17 ± 2.11
Basophils	0.55 ± 0.21

associated with grip strength when *CEBPB* expression is included as an explanatory covariate.

To confirm the microarray result, we re-measured *CEBPB* expression in 100 male InCHIANTI subjects. Very similar results were found plotting tertile of *CEBPB* expression against grip strength (Fig. S1): those in the top expression tertile had higher mean strength compared with those in the bottom tertile (*CEBPB* expression correlation with handgrip strength, $P = 0.048$, excluding five outlier values).

Associations with strength in other muscle systems and with physical performance

We next examined associations between *CEBPB* expression and muscle strength measured at other sites, in models adjusted as previously. There were significant positive associations with measures at the knee, hip, ankle, and shoulder (Table S2).

A critical question is whether the changes in muscle strength seen are of functional significance. Those in the highest tertile of *CEBPB* expression were far less likely to have impaired performance on the Short Physical Performance Battery (SPPB) score (Table S3): the highest expression tertile group had an odds ratio of 0.42 (95% CI, 0.20–0.86; $P = 0.018$) for having impaired performance (SPPB score < 10, 22.35% of the sample). There were trends for the three subscales, with a significant association for walk speed impairment (OR = 0.33; 95% CI, 0.14–0.80; $P = 0.014$).

Muscle strength and physical activity

In *post hoc* sensitivity analyses, we explored whether the association of *CEBPB* expression with muscle strength was explained by higher levels of physical activity. Adjusting for seven levels of physical activity over the previous 1 or 3 years had limited impact on the association between *CEBPB* expression and handgrip, which remained strongly significant ($\beta = 0.13$; 95% CI, 0.06–0.19; $P = 0.001$; and $\beta = 0.16$; 95% CI, 0.09–0.23; $P < 0.001$, respectively).

A potential mechanism underlying the *CEBPB* association with muscle strength

To identify, as far as possible, the likely general mechanisms mediating the *CEBPB* association with muscle strength, we searched the Gene Ontology database (Ashburner *et al.*, 2000) for pathways in which *CEBPB* is involved; this yielded 16 results, most of which are inflammatory-/immune-related, including acute and chronic stages of inflammation, response to stress and wound healing. Gene Ontology does not include a specific pathway explicitly including both *CEBPB* and muscle, or macrophage activation states. A Gene Set Enrichment Analysis (see Experimental procedures), which determines whether sets of biologically related genes are more strongly associated with grip strength than might occur by chance, showed no significant associations (*P*-values > 0.05). A leading alternative pathway involved in muscle repair is *WNT* signaling, but we found no evidence that β-catenin (downstream effector of Wnt) expression was associated with grip strength in our leukocyte samples ($P > 0.05$).

Discussion

Loss of muscle strength is a core feature of aging, and a range of potential local and systemic mechanisms have been proposed to explain it (Lang *et al.*, 2009). Among the more promising is the theory that contraction-induced damage to muscle is common and associated with impaired

Table 2 Probes most closely associated with handgrip strength (natural log kg), with a false discovery rate *q*-value < 20%

Rank	Probe	Gene	Strength coefficient	95% CI	*P*-value	*q*-value
1	*ilmn_1693014*	*CEBPB*	0.20560	0.14 to 0.27	1.03E-06	0.014416
2	*ilmn_1772523*	*TAAR8*	1.06986	0.66 to 1.48	2.46E-05	0.123658
3	*ilmn_1687652*	*TGF-β3*	1.25827	0.76 to 1.75	3.40E-05	0.13609
4	*ilmn_1745583*	*EXOC1*	1.30954	0.78 to 1.84	5.01E-05	0.148728
5	*ilmn_1652417*	*CBWD5*	−1.22926	−1.73 to −0.73	5.38E-05	0.150724
6	*ilmn_1890206*	*CA433556–CD40L*	1.24223	0.73 to 1.76	7.74E-05	0.171274
7	*ilmn_1782050*	*CEBPD*	0.18314	0.11 to 0.26	9.67E-05	0.182561

Models adjusted for age; gender; lifetime pack-years smoked; waist circumference; education; study site; and proportions of leukocyte cell types plus RNA hybridization and amplification batch.

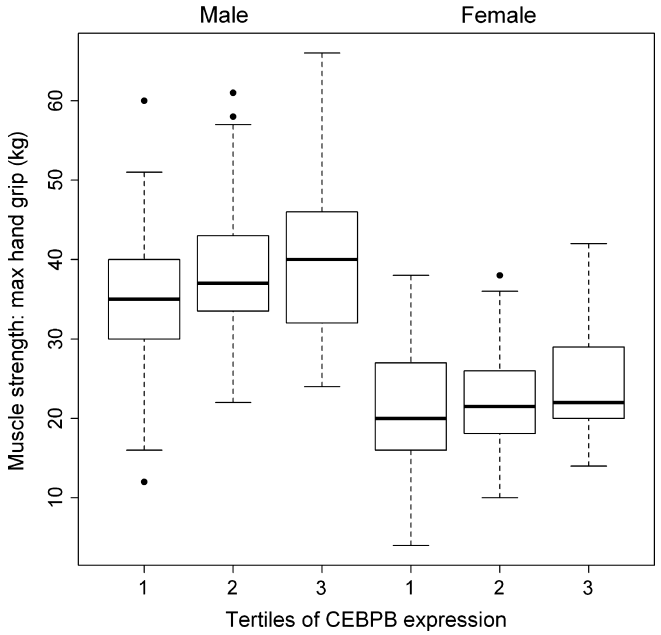

Fig. 2 Demonstrating increased *CEBPB* expression with higher grip strength. Using a box plot and tertiles of *CEBPB* expression, the association with grip strength is clearly visible. *CEBPB* is split by gender because of the natural disparity in muscle strength. Note: for those with 'normal', mid-tertile, and leukocyte percentages. CEBPB, CCAAT/enhancer-binding protein-beta.

Table 3 Regression model–based estimated handgrip strength for men and women aged 70 and with lymphocyte percentage at mean value (30.8%)

	Men		Women	
C\EBP-β expression tertile	Estimated mean strength (kg)	95% Prediction interval (kg)	Estimated mean strength (kg)	95% Prediction interval (kg)
1	35.2	24.1–46.3	20.0	9.1–31.0
2	38.2	37.1–39.4	23.1	22.1–24.0
3	41.2	29.4–53.0	26.0	14.0–37.9

Models adjusted for age; gender; lifetime pack-years smoked; waist circumference; education; study site; and proportions of leukocyte cell types plus RNA hybridization and amplification batch.

repair in old age (Faulkner *et al.*, 1995). Almost every activity of daily living involves lengthening contractions, the majority of which do not induce injury: with age, however, the increasing numbers of highly stretch-susceptible fibers place the muscles of older animals and humans at greater risk (Lynch *et al.*, 2008). A recently published mouse model with reduced *CEBPB* expression after muscle injury prevented the transition from the pro-inflammatory M1- to M2 macrophage-mediated repair and regeneration phases and resulted in sarcopenia-like loss of muscle fibers and fibrosis of muscle tissue (Ruffell *et al.*, 2009). Until now, the relevance of this experimental demonstration to muscle strength in people *in vivo* was unknown.

We set out to test blood leukocyte–derived gene expression associations with muscle strength in an adult human population. We found, for the first time, that increased *CEBPB* expression was strongly associated with greater muscle strength in a human population. We suggest that this is because of increased recruitment of alternatively activated macrophages; that we observe this in peripheral blood leukocytes is not unexpected, as macrophages naturally form a subset of these. By controlling for the varying proportions of the leukocytes from the individual samples, the association between *CEBPB* expression and muscle strength comes to light. Circulating monocytes were not found to be associated with muscle strength; however, the primary source of monocytes during repair are those already residing in the muscle tissue; thus, the circulating monocytes would not necessarily correlate directly with muscle strength.

C/EBP-β is a transcription factor playing a role in 16 pathways classified within the Gene Ontology system (Ashburner *et al.*, 2000); however, we did not find evidence of deregulation of these general (mostly inflammatory) pathways. We also found no evidence that β-catenin (downstream effector of Wnt), an alternative potential explanatory pathway influencing proliferation and differentiation of muscle-residing monocytes (Polesskaya *et al.*, 2003), is differentially expressed. The most likely remaining explanation for our finding appears to be that higher *CEBPB* expression occurs with more effective M2-macrophage-mediated regeneration in muscle tissue; C/EBP-β is involved in both M1 and M2 expression profiles. However, additional activation above basal expression, via CREB, only occurs in M2; IL-10, produced by regulatory macrophages, is dependent upon C/EBP-β-mediated transcription (Liu *et al.*, 2003), as is Arg-1 (produced by wound-healing macrophages; Gray *et al.*, 2005), and others.

Clearly, further work is needed to confirm that the M2-mediated regeneration process is indeed the source of the higher *CEBPB* expression in our blood-derived RNA samples. The regeneration phase after injury is relatively lengthy (8 days typically) compared with the initial pro-inflammatory phase (2 days), and therefore regeneration-related signaling can be expected to predominate in population-level studies of participants unselected with respect to the timing of everyday muscle fiber injury.

C/EBP-β is a transcription factor, and as such, its specificity of action is tightly regulated by phosphorylation and competing cofactors. The precise nature of the promoters that C/EBP-β binds to and activates (i.e., M1 or M2) is dependent on upstream effectors of *CEBPB* expression, such as TGF-β, and on whether C/EBP-β is partnered with pro-inflammatory cofactors such as NF-κB or with anti-inflammatory cofactors such as STAT6 (Fig. 1). We found both TGF-β3 and one of the three isoforms of TGFBr2 were significantly associated with measures of strength ($P = 3.4*10^{-5}$ and $P = 0.037$, respectively). As TGF-β (produced by macrophages after phagocytosis of apoptotic cells; Fig. 1b) attenuates pro-inflammatory signals (Fadok *et al.*, 1998), its up-regulation alongside *CEBPB* is a further indication that an enhanced anti-inflammatory (M2) phenotype is advantageous for maintaining muscle strength.

There is high homology between the C/EBP members; they can form heterodimers (Kinoshita *et al.*, 1992) and substitute for one another to rescue functionality (Jones *et al.*, 2002). However, each is separated functionally by their response to stimuli, their location within the nucleus, and their tissue specificity (i.e., macrophage or muscle cell). C/EBP-δ (which was also associated with muscle strength in our study) has previously been shown to reverse the inactivation of PPAR-γ (Lai *et al.*, 2008); this results in the stimulation of fatty acid oxidation, glycolysis, mTOR activation, and anti-inflammatory actions in macrophages in order to increase hepatic lipogenesis in the liver and adipose tissue (Sag *et al.*, 2008). This is attributed to differing ratios of saturated to unsaturated fatty acids in the blood, which promote either M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotypes, respectively (Kang *et al.*, 2008; Odegaard *et al.*, 2008; Schug & Li, 2009). It is therefore interesting that we found a correlation between *CEBPD* expression and muscle strength in our cohort. This may possibly be due to an effect of *CEBPD* on circulating saturated

fatty acid levels in leaner people, producing a more M2-like response and better muscle repair. Again, this will require further work to elucidate the exact mechanism.

Large-scale gene expression studies are confounded by the high variability between healthy individuals. Results can be affected by a range of factors from ethnicity to employment, including details of blood sample storage (Dumeaux *et al.*, 2010), and therefore we have used a collection procedure designed to stabilize RNA at the point of collection from the participants' veins. We found the proportion of different white cell subtypes to be a significant factor when analyzing *CEBPB* expression. Owing to the nature of the investigation (observing increased *CEBPB* expression hypothesized to be due to, or involved with the causation of, increased anti-inflammatory macrophages), it is not surprising that varying proportions of neutrophils (and lymphocytes) between people influence *CEBPB* expression; however, we found no evidence of interactions between *CEBPB* expression, leukocyte subtype proportions, and muscle strength. In addition, the *CEBPB* expression association with strength was still present in those with 'normal-range' mid-tertile lymphocyte and neutrophil counts. Neutrophils are upstream of macrophages; they are involved in the initial recruitment to sites of muscle injury and amplify the chain of events leading to successful resolution of damage (Tidball, 2005; Tidball & Villalta, 2010).

Along with C/EBP-β, C/EBP-δ, and TGF-β, 4 other probes were associated (genome-wide) with grip strength. These were trace amine-associated receptor 8 (*TAAR8*), EXOcyst Complex component 1 (*EXOC1*), COBW domain-containing protein 5 (*CBWD5*), and cell-surface receptor 40 ligand (*CD40L*). CD40L is associated with neutrophil count and has previously been described to be involved in inflammation (Borcherding *et al.*, 2010). TAAR8 is a receptor for trace amines that are important in neuronal function (Lindemann *et al.*, 2005). EXOC1 and CBWD5 are proteins necessary for exocytic vesicle targeting (Andersen & Yeaman, 2010) and neurotransmission, respectively. CD40L may therefore play a role in M1 macrophage activation, while the remaining genes may reflect improved motor neuron function in people with better muscle strength.

A simple association between *CEBPB* expression and muscle strength would be of limited interest without evidence that *CEBPB expression* is also associated with everyday functioning, impairment, and disability. Lower handgrip muscle strength in 45- to 68-year-old men in the Honolulu Heart Program was predictive of disability 25 years later (Rantanen *et al.*, 1999). The SPPB score is a well-validated and widely used objective measure of functional impairment and is predictive of subsequent disability and mortality (Guralnik *et al.*, 1995). Our finding of an association between higher *CEBPB* expression and a markedly lower risk of impairment on the SPPB suggests that our findings may be of clinical and practical significance.

Given the mouse model demonstrating that increased *CEBPB* expression is necessary for muscle regeneration, it is plausible that the *CEBPB* expression associations reported here with muscle strength reflect a causal relationship, although the statistical association may theoretically reflect an effect rather than a cause. As noted, future work is needed to confirm that higher *CEBPB* expression in peripheral blood–derived samples does indeed reflect greater M2-macrophage-mediated muscle repair. Studies of responses to experimentally induced muscle stresses could chart the temporal response from early reaction to injury through to the later repair phases in people of different ages and strengths. It may be possible to explore the effects of polymorphisms (SNPs) in the implicated genes on expression and strength. Eventually, experimental approaches to enhance *CEBPB* pathway expression and related repair may provide a means of protecting and enhancing aging muscle.

Conclusion

We have presented the first report of the association between raised *CEBPB* expression *in vivo* and greater muscle strength in humans. This may be consistent with mouse model findings that C/EBP-β signaling is critical for the regeneration of muscle fibers after injury. TGF-β, a key initiator and cofactor, is also up-regulated. *CEBPB* expression is also associated with functioning on the SPPB score. Independent replication and mechanistic studies linking *CEBPB* expression in leukocytes to muscle are needed.

Experimental procedures

Samples

InCHIANTI (Ferrucci *et al.*, 2000) is a population-based prospective study in the Chianti area (Tuscany) of Italy. The participants were enrolled in 1998–2000 and were interviewed and examined every 3 years. Ethical approval was granted by the Instituto Nazionale Riposo e Cura Anziani institutional review board in Italy. Participants gave informed consent to participate.

RNA collection, extraction, and the whole-transcriptome scan

Peripheral blood specimens preserving *in vivo* RNA expression were collected in 2008–2009 from 733 participants. RNA was extracted from peripheral blood samples using the PAXgene Blood mRNA kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. This ensures the transcript expression is as near to its *in vivo* levels as possible (Debey-Pascher *et al.*, 2009). Whole-genome expression profiling of the samples was conducted using the Illumina Human HT-12 microarray (Illumina, San Diego, CA, USA) as previously described (Zeller *et al.*, 2010). Data processing was performed using the Illumina and Beadstudio software (Illumina) as previously described (Zeller *et al.*, 2010). All microarray experiments and analyses complied with MIAME guidelines. Data from 698 individuals and 16 571 probes passed our quality control process; 12 subjects were excluded on the basis that their mean signal intensities across all probes with $P \leq 0.01$ were > 3 standard deviations from the cohort mean; probes with < 5% of subjects giving intensities with $P \geq 0.01$ different from background were also excluded.

Handgrip strength

Maximum handgrip strength was measured using a grip dynamometer, recording the maximum force generated (kg) during two attempts with each hand. In our analysis, we used the participant's highest measured grip strength for either hand.

Statistical analysis

Expression data were available for 707 individuals. Seven individuals were excluded following subject-level QC steps, where all intensity data were missing or ± 3 standard deviations from the mean (leaving 698 individuals). The relationship between gene expression and handgrip and other muscle strength was tested mainly using linear regression models with natural log-transformed gene expression level as the dependent variable. Separate regression models were fitted for each of the 16 571 expressed probes. We used the FDR to account for multiple testing. R (statistical computing language) v2.8.1 (The R Foundation for Statistical Computing,

Vienna, Austria) was used for large-scale analyses and STATA v10.1 (Timberlake Consultants, London, UK) for confirmation and additional exploration.

Regression models were adjusted for potential confounding factors on gene expression: age; gender; lifetime pack-years smoked (in five categories: none, less than 20, 20–39, 40 plus years, and missing); waist circumference (as a continuous trait); highest level of education attained (in five categories: none, elementary, secondary, high school, and university/professional); study site [individuals were drawn from a rural village (Greve) and an urban population (Bagno a Ripoli)]; and the proportion of leukocyte cell types (neutrophil %, lymphocyte %, monocyte %, eosinophil %). We also controlled for potential hybridization and/or amplification batch effects in all our analyses.

The SPPB score is a summary of performance on walk speed, time to stand up from the sitting position several times (chair stands), and graded balance tests (Vasunilashorn *et al.*, 2009), and SPPB scores are predictive of disability progression and mortality. Physical activity was classified based on the metabolic equivalent (Ainsworth *et al.*, 1993) approach into: hardly any activity; mostly sitting/some walking; light exercise 2–4 h per week; moderate 1–2 h or light > 4 h per week; moderate exercise > 3 years per week; intense exercise many times per week; and walks 5+ km per day, 5+ days per week.

TaqMan low-density array (TLDA) validation of microarray results

We selected a subsample of 100 male subjects with middle tertile lymphocyte percentages for validation of the *CEBPB* expression data. Total RNA (30–170 ng) was reversed transcribed in 20-μL reactions using the Superscript III VILO kit (Invitrogen, Paisley, UK), according to the manufacturer's instructions. *CEBPB* expression levels for each target transcript were then measured using the TLDA platform (Applied Biosystems, Foster City, CA, USA), using commercially available assay *CEBPB*-Hs00942496_s1. Reaction mixes contained 50 μL 2× TaqMan universal master mix (no AMPerase; Applied Biosystems), 30 μL dH_2O, and 20 μL cDNA template. Cycling conditions were 50°C for 2 min, 94.5°C for 10 min followed by 50 cycles of 97°C for 30 s and 57.9°C for 1 min. *CEBPB* expression was measured in triplicate for each sample. Relative gene expression levels were calculated using the comparative C_t technique (Applied Biosystems, 2001) using the StatMiner relative quantification software developed for analysis of TLDA plates (Integromics, Granada, Spain). Gene expression levels were calculated relative to the geometric mean of the three genes – *COQ2*, *NFKB1,* and *TNF* – whose transcript expression was most stable across all samples, as identified by the GeNorm function of the StatMiner software (Integromics).

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) is an alternative method of analyzing RNA expression data, in which gene sets are specified by the user (can be custom or downloaded from the GSEA site) and the association across the whole set of genes (usually related functionally) is tested against a phenotype (Mootha *et al.*, 2003; Subramanian *et al.*, 2005). We downloaded the 16 Gene Ontology (Ashburner *et al.*, 2000) pathways in which CEBPB is involved, which included 'inflammatory response', 'defense response', 'response to stress', and 'RNA metabolic process'. See Harries *et al.* (2011) for detailed methods.

Acknowledgments

The authors declare no conflict of interest. This analysis was supported by funding from the Peninsula College of Medicine and Dentistry (John Bull Building, Tamar Science Park, Research Way, Plymouth PL6 8BU, UK).

Author contributions

LWH oversaw the validation experiments, interpreted the data, and co-wrote the manuscript. LP carried out the multivariable regression analysis, interpreted the data, and contributed to the manuscript. DH carried out the microarray experiments. RBS carried out the real-time PCR validation of *CEBPB* levels. WH oversaw the statistical analysis and contributed to the manuscript. AS oversaw the microarray experiments. JMG aided in the design of the InCHIANTI study and contributed to the manuscript. SB oversaw the InCHIANTI study and contributed to the manuscript. LF organized the sample cohort and contributed to the manuscript. DM managed the project, interpreted the data, co-wrote the manuscript, and contributed funding.

References

Ainsworth BE, Haskell WL, Leon AS, Jacobs DR Jr, Montoye HJ, Sallis JF, Paffenbarger RS Jr (1993) Compendium of physical activities: classification of energy costs of human physical activities. *Med. Sci. Sports Exerc.* **25**, 71–80.

Andersen NJ, Yeaman C (2010) Sec3-containing exocyst complex is required for desmosome assembly in mammalian epithelial cells. *Mol. Biol. Cell* **21**, 152–164.

Applied Biosystems (2001) Relative quantitation of gene expression. *User Bulletin* **2**, 11–15.

Arnold L, Henry A, Poron Fo, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK, Chazaud B (2007) Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* **204**, 1057–1069.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. *Nat. Genet.* **25**, 25–29.

Borcherding F, Nitschke M, Hundorfean G, Rupp J, Von Smolinski D, Bieber K, Van Kooten C, Lehnert H, Fellermann K, Buning J (2010) The CD40-CD40L Pathway contributes to the proinflammatory function of intestinal epithelial cells in inflammatory bowel disease. *Am. J. Pathol.* **176**, 1816–1827.

Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA (2007) Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science* **317**, 807–810.

Bradley MN, Zhou L, Smale ST (2003) C/EBP{beta} regulation in lipopolysaccharide-stimulated macrophages. *Mol. Cell. Biol.* **23**, 4841–4858.

Carlson BM, Faulkner JA (1989) Muscle transplantation between young and old rats: age of host determines recovery. *Am. J. Physiol.* **256**, C1262–C1266.

Debey-Pascher S, Eggle D, Schultze JL (2009) RNA stabilization of peripheral blood and profiling by bead chip analysis. *Methods Mol. Biol.* **496**, 175–210.

Dumeaux V, Olsen KS, Nuel G, Paulssen RH, Borresen-Dale A-L, Lund E (2010) Deciphering normal blood gene expression variation – the NOWAC postgenome study. *PLoS Genet.* **6**, e1000873.

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest.* **101**, 890–898.

Faulkner JA, Brooks SV, Zerba E (1990) Skeletal muscle weakness and fatigue in old age: underlying mechanisms. *Annu. Rev. Gerontol. Geriatr.* **10**, 147–166.

Faulkner JA, Brooks SV, Zerba E (1995) Muscle atrophy and weakness with aging: contraction-induced injury as an underlying mechanism. *J. Gerontol. A Biol. Sci. Med. Sci.* **50 Spec No**, 124–129.

Ferrucci L, Bandinelli S, Benvenuti E, Di Iorio A, Macchi C, Harris TB, Guralnik JM (2000) Subsystems contributing to the decline in ability to walk: bridging the

gap between epidemiology and geriatric practice in the InCHIANTI study. *J. Am. Geriatr. Soc.* **48**, 1618.
Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris JSM (2005) Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBP[beta]. *Gene* **353**, 98–106.
Guralnik JM, Ferrucci L, Simonsick EM, Salive ME, Wallace RB (1995) Lower-extremity function in persons over the age of 70 years as a predictor of subsequent disability. *N. Engl. J. Med.* **332**, 556–562.
Harries LW, Hernandez D, Henley W, Wood AR, Holly AC, Bradley-Smith RM, Yaghootkar H, Dutta A, Murray A, Frayling TM, Guralnik JM, Bandinelli S, Singleton A, Ferrucci L, Melzer D (2011) Human aging is characterized by focused changes in gene expression and deregulation of alternative splicing. *Aging Cell* **10**, 868–878.
Jones LC, Lin M-L, Chen S-S, Krug U, Hofmann W-K, Lee S, Lee Y-H, Koeffler HP (2002) Expression of C/EBP-beta from the C/EBP-alpha gene locus is sufficient for normal hematopoiesis in vivo. *Blood* **99**, 2032–2036.
Kang K, Reilly SM, Karabacak V, Gangl MR, Fitzgerald K, Hatano B, Lee CH (2008) Adipocyte-derived Th2 cytokines and myeloid PPAR-delta regulate macrophage polarization and insulin sensitivity. *Cell Metab.* **7**, 485–495.
Kinoshita S, Akira S, Kishimoto T (1992) A member of the C/EBP family, NF-IL6-beta, forms a heterodimer and transcriptionally synergizes with NF-IL6. *Proc. Natl Acad. Sci. USA* **89**, 1473–1476.
Lai PH, Wang WL, Ko CY, Lee YC, Yang WM, Shen TW, Chang WC, Wang JM (2008) HDAC1/HDAC3 modulates PPARG2 transcription through the sumoylated CEBPD in hepatic lipogenesis. *Biochimica et Biophysica Acta Mol. Cell Res.* **1783**, 1803–1814.
Lang T, Streeper T, Cawthon P, Baldwin K, Taaffe DR, Harris TB (2009) Sarcopenia: etiology, clinical consequences, intervention, and assessment. *Osteoporos. Int.* **21**, 543–559.
Lindemann L, Ebeling M, Kratochwil NA, Bunzow JR, Grandy DK, Hoener MC (2005) Trace amine-associated receptors form structurally and functionally distinct subfamilies of novel G protein-coupled receptors. *Genomics* **85**, 372–385.
Liu Y-W, Tseng H-P, Chen L-C, Chen B-K, Chang W-C (2003) Functional cooperation of simian virus 40 promoter factor 1 and CCAAT/enhancer-binding protein beta and delta in lipopolysaccharide-induced gene activation of IL-10 in mouse macrophages. *J. Immunol.* **171**, 821–828.
Lynch GS, Faulkner JA, Brooks SV (2008) Force deficits and breakage rates after single lengthening contractions of single fast fibers from unconditioned and conditioned muscles of young and old rats. *Am. J. Physiol. Cell Physiol.* **295**, C249–C256.
Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstråle M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat. Genet.* **34**, 267–273.
Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8**, 958–969.
Nguyen HX, Tidball JG (2003) Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells in vitro. *J. Physiol.* **547**, 125–132.
Odegaard JI, Ricardo-Gonzalez RR, Red Eagle A, Vats D, Morel CR, Goforth MH, Subramanian V, Mukundan L, Ferrante AW, Chawla A (2008) Alternative M2 activation of Kupffer cells by PPAR-delta ameliorates obesity-induced insulin resistance. *Cell Metab.* **7**, 496–507.
Peake J, Della Gatta P, Cameron-Smith D (2010) Aging and its effects on inflammation in skeletal muscle at rest and following exercise-induced muscle injury. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298**, R1485–R1495.
Polesskaya A, Seale P, Rudnicki MA (2003) Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. *Cell* **113**, 841–852.
Rantanen T, Guralnik JM, Foley D, Masaki K, Leveille S, Curb JD, White L (1999) Midlife hand grip strength as a predictor of old age disability. *JAMA* **281**, 558–560.
Robertson TA, Maley MAL, Grounds MD, Papadimitriou JM (1993) The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis. *Exp. Cell Res.* **207**, 321–331.
Ruffell D, Mourkioti F, Gambardella A, Kirstetter P, Lopez RG, Rosenthal N, Nerlov C (2009) A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc. Natl. Acad. Sci. U S A* **106**, 17475–17480.
Sag D, Carling D, Stout RD, Suttles J (2008) Adenosine 5′-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J. Immunol.* **181**, 8633–8641.
Schug TT, Li X (2009) PPAR-delta-mediated macrophage activation: a matter of fat. *Dis. Model. Mech.* **2**, 421–422.
St Pierre BA, Tidball JG (1994) Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension. *J. Appl. Physiol.* **77**, 290–297.
Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl Acad. Sci. USA* **102**, 15545–15550.
Tidball JG (2005) Inflammatory processes in muscle injury and repair. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**, R345–R353.
Tidball JG, Villalta SA (2010) Regulatory interactions between muscle and the immune system during muscle regeneration. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298**, R1173–R1187.
Vasunilashorn S, Coppin AK, Patel KV, Lauretani F, Ferrucci L, Bandinelli S, Guralnik JM (2009) Use of the short physical performance battery score to predict loss of ability to walk 400 meters: analysis from the InCHIANTI study. *J. Gerontol. A Biol. Sci. Med. Sci.* **64A**, 223–229.
Villalta SA, Nguyen HX, Deng B, Gotoh T, Tidball JG (2009) Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. *Hum. Mol. Genet.* **18**, 482–496.
Zeller T, Wild P, Szymczak S, Rotival M, Schillert A, Castagne R, Maouche S, Germain M, Lackner K, Rossmann H, Eleftheriadis M, Sinning CR, Schnabel RB, Lubos E, Mennerich D, Rust W, Perret C, Proust C, Nicaud V, Loscalzo J, Hubner N, Tregouet D, Munzel T, Ziegler A, Tiret L, Blankenberg S, Cambien F (2010) Genetics and beyond – the transcriptome of human monocytes and disease susceptibility. *PLoS ONE* **5**, e10693.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 Top 250 microarray probes (genes) associated with handgrip strength (kg).

Table S2 Regression based estimated of C/EBP-ß expression associations with measures of strength.

Table S3 Odds ratio for having impaired performance on performance scores, comparing those with top tertile CEBP-ß expression to lowest tertile expression.

Fig. S1 Microarray Validation – Hand Grip strength by tertiles of C\EBP-β expression measured by TLDA, for 100 men with middle tertile lymphocyte and neutrophil percentages.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.