SUPPLEMENTARY MATERIALS AND METHODS

1. Patients

Retrospective formalin-fixed paraffin-embedded tissue was obtained from a cohort of 150 samples comprising 29 benign melanocytic naevi and 121 primary melanomas of AJCC disease stages I or II at the time of diagnosis and excision at the Royal Victoria Infirmary, Newcastle-upon-Tyne between January 2003 and May 2005. Ethical permission for the study was obtained from the Newcastle and North Tyneside research ethics committee (REF; 08/H0906/95) with written, informed patient consent obtained as appropriate, and the study performed in accordance with the Declaration of Helsinki Principles. The sample cohort was selected based on pre-study power calculations following pilot data to provide a 95% power to detect a correlation (r) of ≥ 0.2 with p62 expression at the $P \leq$ 1E-05 level. These power calculations revealed 24 samples were required in each eventual AJCC disease subgroup (benign naevi, AJCC stage I, II and combined III/IV; 96 samples in total). Tumour subtypes were limited to primary cutaneous superficial spreading or nodular melanomas, with the exclusion of acral or lentigenous subtypes. Clinical staging was based on the 2009 AJCC clinical staging criteria. Data were correlated with clinical outcome after a minimum of seven years follow-up using case-note examination, allowing the correlation of p62 expression in the primary tissue sample with the time to development of first metastasis (disease free survival; DFS) or time to death from melanoma (melanoma specific mortality; MSM). Reporting of the data was performed in line with the REMARK guidelines for tumour marker prognostic studies (McShane et al, 2005).

McShane LM, Altman DG, Sauerbrei W, *et al.* (2005). Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK). *J Natl Canc Inst* 97:1180-1184

.

2. Immunohistochemistry

Immunohistochemistry for p62 and MelanA expression were carried out as previously described (Hiscutt et al, 2010). p62 was detected with monoclonal anti-p62 (Santa Cruz Biotechnology Inc. USA; 1:50 with antigen retrieval performed using 0.1M Tris-HCl pH 9), and Melan A with monoclonal anti-Melan A (Abcam, USA; 1:500; antigen retrieval with 0.1M Tris-HCl pH 7.6), both for 1 hour at room temperature and primary antibody binding detected with mouse IgG ABC Elite Vectastain Kit (Vector Laboratories Inc., Burlingame, USA) developed with Vector VIP (Vector Laboratories).

3. Evaluation of NRAS and BRAF activating mutations in the patient cohort

DNA was extracted from FFPE tissue blocks using QIAamp® DNA FFPE Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Immediate tissue sections to those taken for routine histology were taken for DNA extraction. PCR amplification and sequencing of the two most common *NRAS* codon-61 Single Nucleotide Polymorphisms (SNPs) on exon 2: *NRAS* Q61K and Q61R; as well as the SNPs for *BRAF* V600D and V600E mutations were performed using custom made primer and probes as previously described (Hiscutt et al, 2010). SNP analysis revealed the presence of 12 *NRAS* Q61R, 7 *NRAS* Q61K, 48 *BRAF* V600E mutations and 54 samples wild-type for all tested mutations. No *BRAF* V600D mutations were found in the study population.

Hiscutt EL, Hill DS, Martin S, *et al.* (2010). Targeting X-Linked Inhibitor of Apoptosis Protein to Increase the Efficacy of Endoplasmic Reticulum Stress-Induced Apoptosis for Melanoma Therapy. *J Invest Dermatol* 130:2250-2258

4. Immunohistochemical analysis and p62 quantification

The mean percentage positively stained cells was derived by the analysis of up to ten representative 20x magnification microscope fields of vision and expressed as an overall mean percentage of cells expressing p62, using Leica QWin image analysis software (Leica Microsystems). IHC analysis was validated using FFPE cells of known p62 expression, and inter-assay variability assessed by repeated staining of positive and negative 'control' tumour samples which demonstrated a near identical staining pattern and reproducible percentage of cellular staining positivity. Tumour tissue was confirmed by the expression of Melan A in tissue from a matched serial section (Hiscutt et al, 2010) and microscopic analysis by the study pathologist. Although blinding for the diagnosis and major histological criteria was impossible without compromising selection of areas for photography, both assessors (study Dermatologist and Pathologist) were blinded to eventual disease outcome.

5. Patient Demographics

The 2009 AJCC staging criteria was used to define the clinical stage of disease of the 150 patient cohort at diagnosis, revealing 29 benign melanocytic naevi, 67 AJCC stage I, and 54 AJCC stage II tumours. Patient outcome was determined after a minimum of seven years follow-up using case note examination. Disease recurrence was defined by the time to first radiological or tissue diagnosis of metastatic disease (nodal or systemic) from the point of initial primary tumour excision. Further patient demographic data is outlined in Table S1.

Hiscutt EL, Hill DS, Martin S, *et al.* (2010). Targeting X-Linked Inhibitor of Apoptosis Protein to Increase the Efficacy of Endoplasmic Reticulum Stress-Induced Apoptosis for Melanoma Therapy. *J Invest Dermatol* 130:2250-2258

.

STATISTICAL ANALYSIS

Data were expressed as median expression levels between groups (all *P* values presented are unadjusted for multiple testing). Independent group analysis between naevi and melanoma, as well as localised and metastatic disease (eventual AJCC stage I/II versus eventual AJCC stage III/IV disease) was determined by Mann-Whitney *U* (a non-parametric equivalent of a non-paired t-test). The Kruskal-Wallis method (a non-parametric analogue of a one-way ANOVA) was used for analysis of variance between AJCC cohorts. Difference between “high” and “low” p62 expression levels for survival curve analysis was tested using the Wilcoxon Signed Rank Test (a further non-parametric analogue of the t-test). Univariate survival curves and subsequent Log-rank (Mantel-Cox) survival analysis was undertaken using R 2.15.0 (R Foundation for Statistical Computing). Multivariate Cox proportional hazards models (a form of survival analysis which explicitly takes into account the effects of possible confounders) were fitted with a 'forwards-backwards' iterative approach beginning with lower order terms to select interaction terms, using the *coxph* & *anova* functions in R 2.15.0 (R Foundation for Statistical Computing) and high/low p62 expression as a stratifying variable. Covariates considered were: age, sex, dichotomized Breslow depth (<2mm), ulceration status, stage at diagnosis and mutation status. A *P* value <0.05 was considered significant throughout.

Interactions higher than second order were not considered since no second order interaction term was found to provide a significant improvement the baseline model. The final model obtained for the multivariate time to metastasis analysis was:

Surv ~ sex + breslow.group + mutation + strata(p62)

Table S1: Demographic data of study cohort patient samples

Patient Demographics	
Melanoma	
Number of patients	121
Male: Female	62:59
Median age at diagnosis (range)	56 (1-87)
AJCC Stage at diagnosis	
I	67
II	54
Eventual AJCC Stage	
I	57
II	23
III/IV	41
***NRAS/BRAF* Mutational status**	
***NRAS* mutant**	19
***BRAF* mutant**	48
Wild-type	54
Melanoma subtype	
Superficial Spreading	97
Nodular	24
Median Breslow Depth (range)	1.6 mm (0.3-19)
Median Clark's Level (range)	III (II-V)
Number of Ulcerated Primary Tumours	29 (24%)
Number of melanoma related deaths	25 (21%)
Melanocytic Naevi	
Number of patients	29
Male:Female	12:17
Median age at diagnosis (range)	36 (11-79)
Subtype	
Compound	5
Intradermal	24
Site	
Head and neck	12
Trunk	11
Limbs	6

.

Table S2. Univariate analysis of hypothesised risk factors for Disease Free Survival and Melanoma Specific Mortality using Log-rank (Mantel-Cox) calculations. Analysed values for Breslow depth and age were determined using median values for each variable within the study cohort.

Univariate Analysis Disease Free Survival			
Variable	**Hazard Ratio**	**95% Confidence Interval**	***P* value**
p62 expression <20%	1.66	1.03 to 2.69	**0.03**
Breslow depth >1.6mm	4.43	2.28 to 8.62	**<0.0001**
Ulcerated primary tumour	2.34	1.49 to 3.67	**<0.0001**
Male sex	1.96	1.02 to 3.77	**0.04**
NRAS/BRAF mutant	1.61	1 to 2.61	0.05
Age >56 years	1.1	0.7 to 1.74	0.67
Univariate Analysis for Melanoma Specific Mortality			
p62 expression <20%	1.5	0.83 to 2.74	0.18
Breslow depth >1.6mm	3.45	1.61 to 7.39	**<0.0001**
Ulcerated primary tumour	1.9	1.06 to 3.43	**0.03**
Male sex	3.26	1.29 to 8.22	**0.01**
NRAS/BRAF mutant	1.28	0.72 to 2.31	**0.4**
Age >56 years	1.2	0.66 to 2.18	0.54

Table S3: Multivariate analysis of hypothesised risk factors for Disease Free Survival using Cox Proportional Hazards model.

Multivariate Analysis Disease Free Survival			
Variable	**Hazard Ratio**	**95% Confidence Interval**	**P value**
Male Sex	2.001	0.9968-4.015	0.05
Breslow depth >= 2mm	3.361	1.9625-5.755	1E-5
NRAS/BRAF Mutant	1.4797	0.7034-3.113	0.30

.

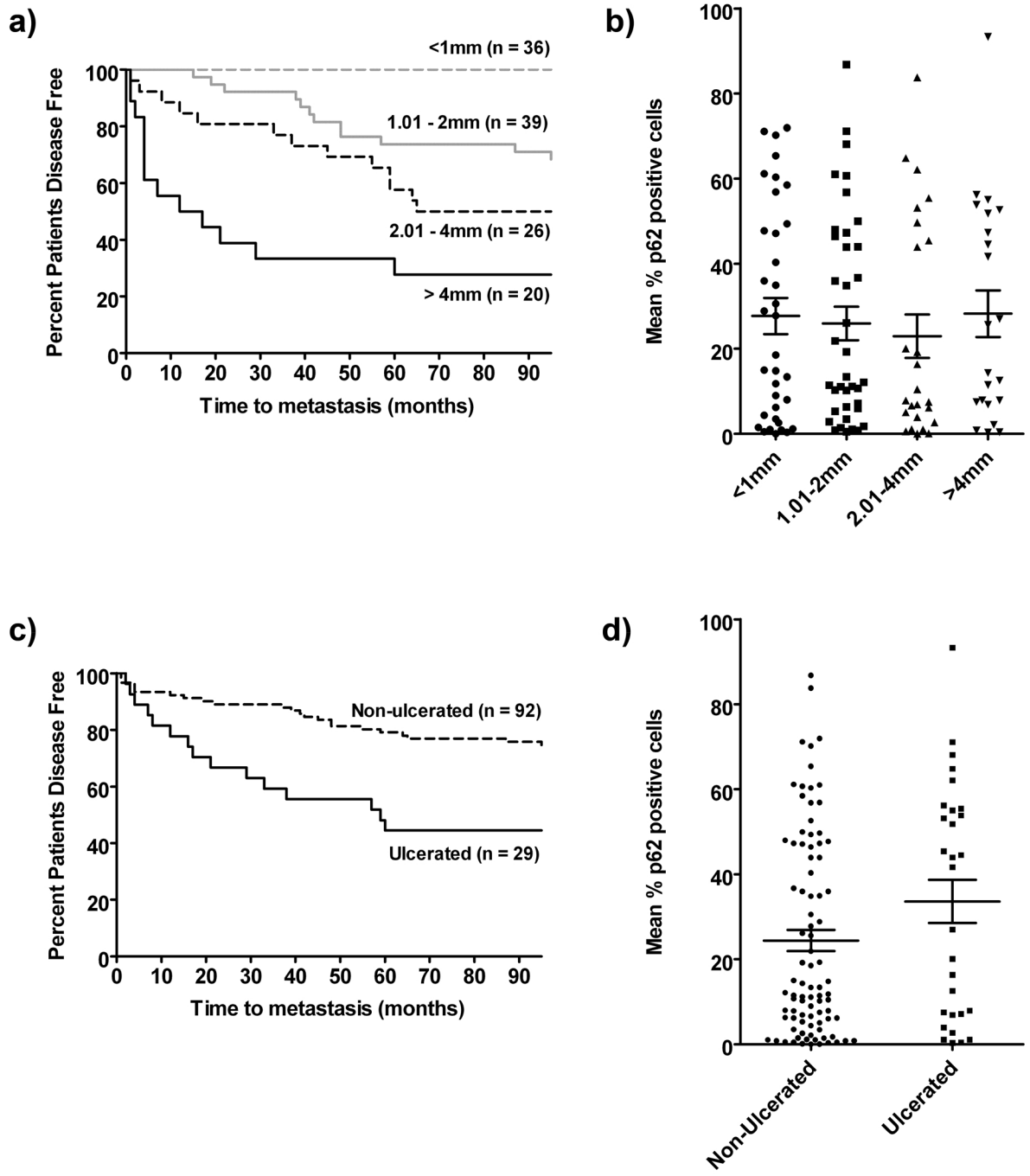

Figure S1

(a) Univariate analysis of Breslow depth revealed a stepwise increased risk of metastases as Breslow depth increases (Log-rank (Mantel-Cox) ***P*** < 0.0001). (b) There was no significant difference in median p62 expression between tumours of differing Breslow depth (Kruskal-Wallis P = 0.77). (c) Univariate analysis of ulceration status revealed a significant increased risk of metastasis in ulcerated primary tumours compared to non-ulcerated (Log-rank (Mantel-Cox) P = 0.0004, HR 4.02 (95% CI 1.77 - 9.13). (d) There was no significant difference in p62 expression between ulcerated and non-ulcerated primary tumours (Mann-Whitney U, P = 0.13)